Universidade de Lisboa

Faculdade de Farmácia

GlcNAc as a therapeutic and cosmetic agent: topical formulation delivery design

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Abstract

Glucosamine and its derivatives are commonly used in the form of oral supplements for the treatment of osteoarthritis, often in combination with chondroitin sulphate. Recently, there have been studies with N-acetyl glucosamine (GlcNAc) which is a more stable form of glucosamine for topical formulations.

Due to its anti-oxidant and anti-inflammatory properties, GlcNAc can be used in the therapy of psoriasis and degenerative joint diseases and with less side effects compared to non-steroidal anti-inflammatory drugs (NSAIDs). Glucosamine has also been proposed to be used in anti-cancer therapy, dementia disease control and in the antimicrobial field. Since glucosamine is the precursor of the hyaluronic acid, when applied topically it improves skin hydration and contributes to an anti-ageing effect and is also effective in the treatment of hyperpigmentation. GlcNAc, which has low price and high availability in the market, is suitable for topical formulations due to its low molecular mass, satisfactory solubility in hydrophobic solvents and high potency in low concentrations.

This work incorporates the review of the therapeutic and cosmetic uses of glucosamine as well as the development of a topical formulation with GlcNAc. The aim was to optimise the amount of GlcNAc that penetrates the skin by maximising its bioavailability so as to counter the low bioavailability of the oral route and obtain therapeutic results faster. The formulations were prepared according to the Formulating for Efficacy theory and tested *in vitro* in vertical glass Franz diffusion cells using Tuffryn® and Strat-M® membranes. Since GlcNAc has a low permeability coefficient, chemical enhancers such as glycerol and propylene glycol were added to the formulations. The results obtained demonstrated that the permeation of GlcNAc was not linear. Therefore, the Higuchi model was applied, and it was observed that it did not fit the results of the entire experiment (4 h) because the permeation of GlcNAc decelerated at 0.5 h. A possible reason may have been a crystallisation effect of GlcNAc that started at 0.5 h of the experiment, due to the evaporation of the water and the faster permeation of the chemical enhancers. The formulation of glycerol, propylene glycol and water is the one that presented the best results in the Strat-M® membrane, which is the more restrictive membrane of the two used in the present work. However, it was not able to surpass the crystallisation effect, suggesting that chemical enhancers may not be the best option for the topical delivery of GlcNAc. A possible alternative could be nanostructuring GlcNAc, so that it would permeate through the skin by the intercellular spaces and by the transcellular pathway, and in this way avoid the crystallisation effect.

Keywords: GlcNAc, therapeutic, cosmetic, skin permeation, Formulating of Efficacy

Resumo

A glucosamina e os seus derivados são amino açúcares que participam em várias funções bioquímicas chave. Eles são precursores de glicosaminoglicanos e têm um bom perfil de segurança. Comummente são utilizados na forma de suplemento oral, frequentemente em combinação com o sulfato de condroitina no tratamento da osteoartrite. Recentemente foram desenvolvidos vários estudos com a N-acetil glucosamina (GlcNAc) que é uma forma mais estável da glucosamina para o uso em formulações tópicas.

Devido às suas propriedades antioxidantes e anti-inflamatórias o GlcNAc pode ser utilizado no tratamento da psoríase e nas doenças degenerativas das articulações e com menos efeitos adversos que os anti-inflamatórios não esteroides (AINEs). Também foi proposto o uso da glucosamina na terapêutica contra o cancro, no controlo da doença demencial e na área antimicrobiana. Outro alvo da glucosamina tópica é o seu uso na cosmética. Uma vez que a glucosamina é precursora do ácido hialurónico, quando aplicada topicamente contribui para a hidratação da pele e para o efeito antienvelhecimento.
Para além disso, vários ensaios clínicos indicam que o GlcNAc é eficaz no tratamento da hiperpigmentação, constituindo uma alternativa à hidroquinona que recentemente foi provada ser cancerígena quando usada por longos períodos e em altas concentrações.
O GlcNAc é apropriado para formulações tópicas devido à sua potência em baixas quantidades, baixa massa molecular e lipofilicidade. Outras características que fazem do GlcNAc um bom candidato para formulações tópicas são o seu baixo preço e a elevada disponibilidade no mercado.

Esta monografia incorpora a revisão do uso terapêutico e cosmético da glucosamina, bem como o desenvolvimento de uma formulação tópica de GlcNAc. O objetivo é otimizar a quantidade de GlcNAc que penetra na pele, maximizando a sua biodisponibilidade de modo a contrariar a baixa disponibilidade da via oral e obter resultados terapêuticos mais rapidamente.
As formulações foram preparadas de acordo com a teoria “Formulação para Eficácia” e foram testadas *in vitro* em células de difusão Franz verticais usando membranas de Tuffryn® e Strat-M®. Uma vez que o GlcNAc possui um coeficiente de permeabilidade muito reduzido, a sua velocidade de permeação é muito baixa, tornando necessária a adição de potenciadores químicos como o glicerol e o propileno glicol à formulação. Para isso foram devolvidas várias formulações com glicerol e água em diferentes proporções, uma formulação só com água e uma mistura de glicerol, propileno glicol e água. Todas as formulações têm em comum o GlcNAc a 2% e o carbómero de Carbopol a 0.5%. No geral, os resultados obtidos nas duas

membranas demonstram que a libertação de GlcNAc não é linear ao longo do tempo. Deste modo, os resultados foram analisados pelo método Higuchi e verificou-se que o método de Higuchi não se adequava a todo o tempo da experiência (4 horas), mas tinha que ser segmentado nos intervalos de 0 – 0.5 h e de 0.5 – 4 h, sugerindo que há uma mudança de comportamento de libertação de GlcNAc a partir da meia hora da experiência. A explicação mais provável é a cristalização do GlcNAc dentro da membrana devido à evaporação da água e devido à permeação dos potenciadores químicos para o compartimento recetor da célula de Franz. A formulação que apresenta melhores resultados na membrana de Strat-M®, ou seja, a membrana mais restrita, é a mistura de glicerol, propileno glicol e água. No entanto, esta formulação não consegue superar o efeito de cristalização do GlcNAc sugerindo que o uso de potenciadores químicos não é a melhor opção para a libertação tópica de GlcNAc.
Uma possível alternativa consiste na nanoestruração do GlcNAc de forma a que este possa permear pelos espaços intercelulares e transcelulares de modo a evitar a sua cristalização na membrana.

Palavras-chave: GlcNAc, terapêutica, cosmética, permeação, “Formulação para Eficácia”

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1. N-acetyl glucosamine

N-acetyl-D-glucosamine or 2-(acetylamino)-2-deoxy-D-glucose, usually known by GlcNAc or NAG is an acetylated amino sugar of glucosamine and is present in the chitin of the shellfish, in human glycosaminoglycans, like hyaluronic acid and in murein, a constituent of the bacterial cell wall. In chitin, N-acetyl-D-glucosamine is the monomeric unit establishing 1,4 – β linkages and in hyaluronic acid it establishes β-glycosidic bonds with D-glucuronic acid.

In humans, it has its origin in D-glucosamine, the precursor of all the amino sugars, after being acetylated by N-acetyl transferase (1).

Currently, both glucosamine and GlcNAc are being used as therapeutic and cosmetic agents (figure 1) (2).

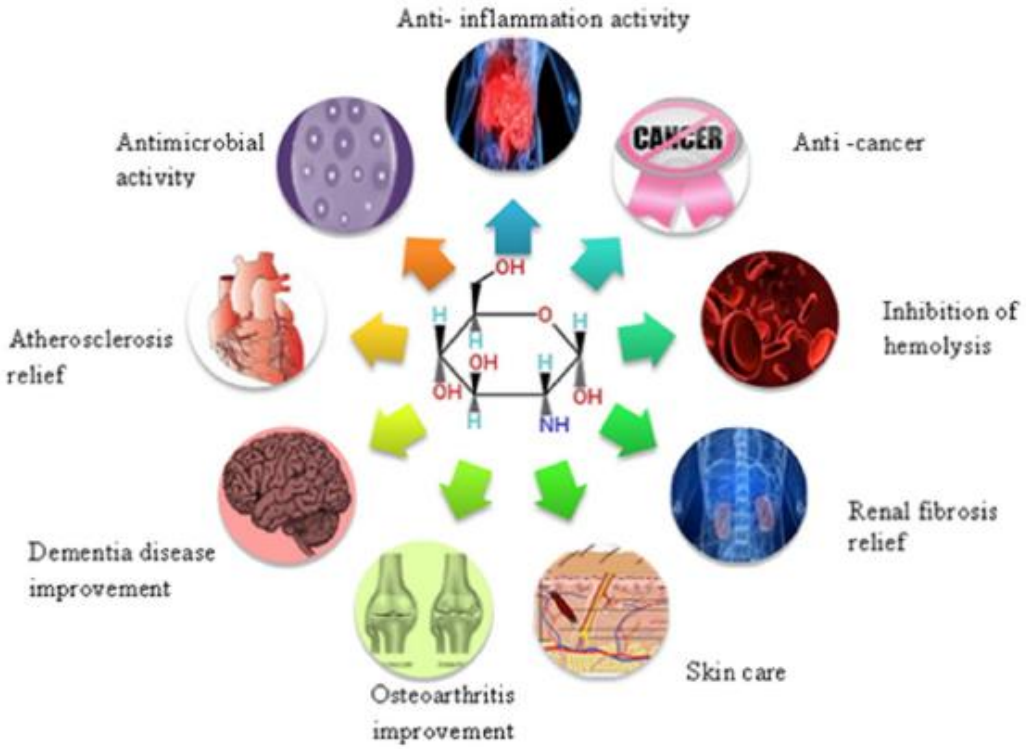


Figure 1 - The applications of N-Acetyl Glucosamine (2).

The therapeutic use of both compounds is relevant in several pathologies due to their capacity to suppress the inflammatory process, by inhibiting the Nuclear Factor-κB, especially in diseases like rheumatoid arthritis. In the market, it is possible to find oral supplements of glucosamine in the forms of N-acetyl glucosamine, glucosamine hydrochloride and glucosamine sulphate (2).

The main source of N-acetyl glucosamine is through chitin hydrolysis, either chemical or enzymatic, as chitin is available in large quantities in the nature. GlcNAc can also be obtained from the microbial fermentation of chitin and through genetically engineered *Escherichia coli* (1).

Glucosamine pharmacokinetics information indicates that when taken orally, in a single daily dose of 1500 mg, nearly 90% of the dose taken is absorbed in the gut, has an elimination half-life of 15 h and it is excreted in the urine and faeces, but the most part, almost 50%, is exhaled. On the other hand, GlcNAc pharmacokinetics shows that it is mostly excreted in the urine (2).

Toxicological tests were performed in humans and indicate that after an administration of 20 g of GlcNAc given intravenously there is no toxicity and no alteration of the glucose levels in the blood. Also, there is no alteration in glucose metabolism and in insulin resistance (3).

Potential side effects can occur in patients with shellfish allergy, as GlcNAc is obtained from crustaceans, and when it is given in the form of salt (e.g. glucosamine sulphate and glucosamine hydrochloride) as it can increase blood pressure.

Therefore, GlcNAc is a safe substance that can be given orally, intravenously or topically, but in a long term, special populations such as diabetic and hypertensive must be carefully monitored (1,4).

1.1. GlcNAc as a therapeutic agent

1.1.1. Anti-inflammatory action

Several studies and clinical trials prove the use of glucosamine as an anti-inflammatory agent that can be used in joint disorders such as osteoarthritis, rheumatoid arthritis, joint damage and degenerative joint disease (DJD). All of these conditions are characterised by a local inflammation and a decrease in the quality of the patient's life (1,4).

Studies with glucosamine sulphate in a dose of 1500 mg per day during a period of three years showed a decrease in pain and an increase in the mobility of the joints. This was evaluated through the Lequesne index, which is an assessment of pain, function and mobility of the joints in patients with osteoarthritis (2).

The anti-inflammatory action is due to glucosamine's ability to inhibit the Nuclear Factor- κB activation as shown in figure 2. It prevents the translocation of P_{65} and P_{50} to the nucleus and factors involved in the inflammatory action such as TNF-α and INF-γ cannot be transcript. Without the transcription of TNF-α and INF- γ, the CD54 gene expression is decreased, ultimately resulting in a decrease of T-cells (2).

Also, a study accomplished by Jeong et al. demonstrated that glucosamine can link directly to transglutaminase 2 (TGase 2) and prevent NF-κB activation. Glucosamine is also responsible for the inhibition of interleukin-1β, which is a pro-inflammatory cytokine involved in the inflammatory process that activates NF- κB (2,5).

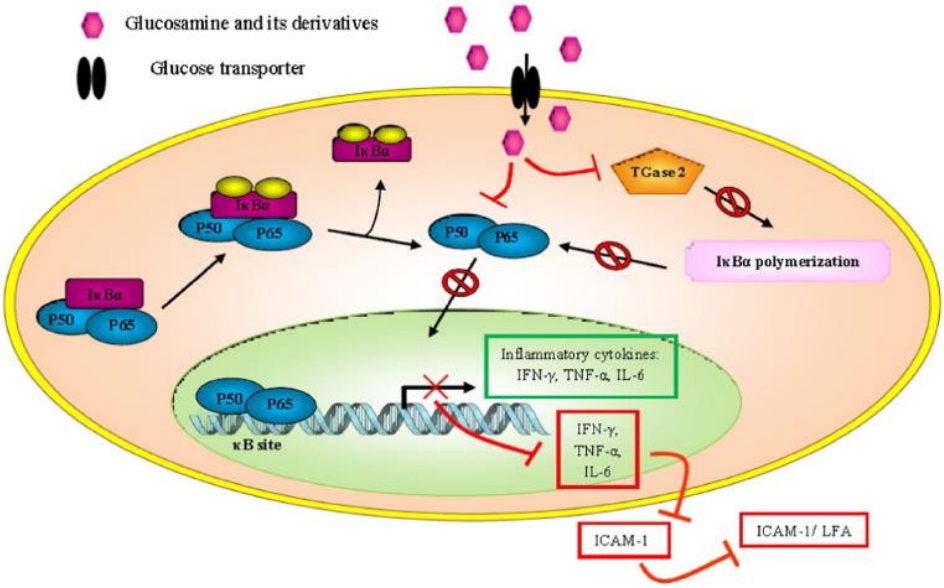


Figure 2 - The inhibition of the inflammatory pathway by glucosamine. Glucosamine enters the cell through the glucose transporter and inhibits the NF-κB activation either by blocking transglutaminase 2 (TGase 2) or by inhibiting the P_{65}-P_{50} complex (2).

Glucosamine was compared with ibuprofen and both had the same efficacy. Although ibuprofen has a faster start of action, glucosamine is better tolerated with the same percentage of adverse reactions as placebo (6).

1.1.2. Chondroprotective action

Besides the anti-inflammatory action of glucosamine that is useful in joint disorders, glucosamine can also improve joint mobility as it decreases extracellular matrix degradation and improves the cartilage matrix (2,4).

Thereby, the reduction in the expression of COX-2, MMP enzymes and the pathways they promote results in a decrease of glycosaminoglycan's degradation, alongside an increase in hyaluronic acid production, which improves the cartilage matrix, relieving the symptoms of joint disorders (7).

Comparisons of the several forms of glucosamine available showed that these effects are most observed in glucosamine sulphate rather than glucosamine hydrochloride, which suggests that maybe the sulphate derivative has actions of its own. More studies need to be performed to fully understand the action of glucosamine sulphate (6).

Also, studies using N-acetyl glucosamine showed that it increases the glucose uptake and enhances the synthesis of glycosaminoglycans and hyaluronic acid more efficiently than glucosamine (4).

A study done by Peterson et al. evaluated the levels of C-telopeptide of type 2 collagen (CTX-II), a marker of collagen degradation eliminated in the urine, and the serum levels of cartilage oligomeric matrix protein (COMP), an element of the cartilage matrix. These two markers are normally increased in patients with osteoarthritis. The levels were evaluated before and after a treatment with glucosamine combined with physical activity and a treatment with ibuprofen,

and the results showed a decrease in the levels of CTX-II in the urine and of COMP in the serum when using glucosamine. Ibuprofen did not seem to exercise a role in cartilage matrix protection, only reducing pain (8).

1.1.3. Atheroprotective activity

In several studies, glucosamine was proved to have a cardioprotective action, preventing ischaemia/reperfusion injury as GlcNAc binds to the hydroxyl groups of serine or threonine residues, in a process mediated by O-GlcNAc transferase, and originates O-linked- N-acetyl glucosamine (O-GlcNAc), a protein that has a major role in the protection of cardiomyocytes (2).

Both glucosamine and O-GlcNAc decrease the expression of NF-κB and thereafter the levels of TNF-α, IL-6 and ICAM-I also decrease, as these factors are part of the systemic inflammatory response. O-GlcNAc has the ability to increase the levels of mitochondrial Bcl-2, which is responsible for the inhibition of the apoptosis of heart tissue cells and thus increase the cardiomyocytes' survival (9).

The administration of glucosamine after a trauma-haemorrhage in a rat performed by Yang et al. showed that the increase in the O-GlcNAc levels promote the Ca^{2+} balance by inhibiting angiotensin II that is responsible for the increase of Ca^{2+} inside the cells during cardiac injury (2,9).

As glucosamine promotes the stabilisation of calcium levels it reduces the incidence of ventricular fibrillation. It is noteworthy that the concentrations of glucosamine necessary to induce cardioprotective effects are significantly higher than those used in the supplements for osteoarthritis (1500 mg) (10).

Glucosamine also has a potent action in atherosclerosis, reducing the inflammation of the arterial blood vessels for several reasons. The first is that it increases perlecan, which is the major constituent of vascular heparan sulphate proteoglycans (HSPGs) that are usually reduced in atherosclerosis. The increase in perlecan decreases the number of monocytes that bind to the endothelial cells and decreases the proliferation of smooth muscle cells. The second reason is the reduction in LDL retention as they depend on the length of the glycosaminoglycan that is attached to the binding proteoglycan, and glucosamine induces a decrease in the glycosaminoglycan's chain length. Besides these factors, markers that evaluate inflammation, such as IL-6 and C-reactive protein, are reduced, which suggests a greater protection of the aorta tissue (2).

1.1.4. Antioxidant action

There are numerous reports that show the antioxidant activity of glucosamine. Glucosamine can protect several molecules from oxidation induced by hydroxyl radicals as it inhibits NADPH oxidase and, therefore, decreases the number of reactive oxygen species (ROS). It can also reduce lipid/protein peroxidation and increase the cell survival under oxidative stress, as it prevents the reduction of glutathione in the cytosol of erythrocytes (2).

1.1.5. Anti-cancer activity

Glucosamine has the potential to be used as an agent in cancer therapy in a concentration and time dependent manner as it delays the rate of tumour growth without affecting the development of healthy tissue (2).

The major reason for its anti-cancer activity is the decrease of N-linked glycosylation (N-glycosylation), an important process for the stabilisation of proteins and their functions, in the newly synthesised proteins. In this way, several proteins involved in tumour activity become disabled. One of these proteins is gp130, that after N-linked glycosylation undergoes a reduction in molecular weight, which in turn decreases the phosphorylation of STAT3, a member of the STAT family that promotes angiogenesis and cancer cell survival. Another consequence of the inhibition of N-glycosylation in gp130 is the reduction of the binding capacity of IL-6 to the cells and suppression of the IL-6/JAK2/STAT3 signalling pathway. Other pathways that require N-glycosylation are AKT and ERK1/2 and both are suppressed by glucosamine (11).

In several reports, glucosamine demonstrated ability to promote cell cycle arrest at G1 phase and induce apoptosis by several mechanisms (autophagy, apoptotic inhibition of proteasomal activity and activation of caspase-3). As tumours have been associated with chronic inflammation, the anti-inflammatory action of glucosamine is important to inhibit IL-1, TNF-α and NF-κB, avoid cancer initiation and the posterior steps, such as angiogenesis and metastasis (2).

1.1.6. Dementia disease improvement

Neurodegenerative diseases are characterised by inflammation and oxidative stress in the brain tissue. The anti-inflammatory and antioxidant properties of glucosamine make it a suitable candidate to delay the progression of neurodegenerative diseases such as Alzheimer.

Studies done in rat brains have shown a decrease in the stroke volume after an intraperitoneal injection of glucosamine. Glucosamine reduced the levels of inflammatory agents like IL-1β, TNF-α and COX-2 (2).

Also, glucosamine suppressed the phosphorylation of P_{65} that is induced by LPS, which is involved in chronic inflammation, and reduced the levels of NF-κB (12).

These results were observed only when glucosamine was used and not with its derivatives. However, further studies must be performed to evaluate the complete role of glucosamine in the brain tissue (2).

1.1.7. Kidney diseases relief

In vitro and *in vivo* studies performed with glucosamine hydrochloride have shown that it can reduce the fibrogenesis of the kidney by inhibiting the TGF-β/Smad pathway. The reduction of TGF-β expression is due to the inhibition of the glycosylation of type II TGF-β receptor and leads to a decrease in protein synthesis in the matrix and an increase of the catabolic activity of the MMP family and plasmin (2).

Another kidney condition that is improved is renal ischaemia as glucosamine hydrochloride promotes GRP78 activity. GRP78 is a chaperon with anti-apoptotic properties and the raise of its expression increases the survival of cells under hypoxic conditions (2).

1.1.8. Antimicrobial activity

In several studies glucosamine sulphate proved to have antimicrobial activity against Gram-positive and Gram-negative bacteria (2).

The antimicrobial mechanism is not fully understood, but is suspected to be related to the free amino groups of the glucosamine sulphate ring that disrupt the cell wall in a mechanism similar of the *para*-aminobenzoic acid (PABA) and sulphonamide (13).

Other studies were performed with glucosamine hydrochloride and an inhibition of the microbial growth was observed in four types of microorganisms responsible for the spoilage of food (14).

More research on this topic needs to be performed to clarify the antimicrobial activity of the derivatives of glucosamine.

1.2. GlcNAc as a cosmetic agent

1.2.1. Wrinkle reduction

With ageing, the amount of hyaluronic acid produced by fibroblasts does not decrease significantly, but its distribution does. Hyaluronic acid becomes more bonded to the proteins in the upper epidermal layers and its ability to hold water decreases, which explains the loss of elasticity of the skin and the formation of wrinkles. Furthermore, the collagen becomes cross linked, which in turn makes the production of more collagen difficult. These processes can be accelerated with UV radiation (14).

GlcNAc, one of the precursors of hyaluronic acid, when applied to the skin increases the production of proteins by the fibroblasts and peritoneal mesothelial cells. This process is not caused by the increase of hyaluronan synthase activity, but by a decrease in the inflammation of the peritoneal mesothelial cells (15).

In the study performed by A. Polubinksa et al. a culture of fibroblasts treated with GlcNAc produced more hyaluronic acid and collagen and produced less interleukin-6 compared with the fibroblasts without treatment.

Further studies are needed to determine the type of collagen produced and the exact changes that GlcNAc exerts on the fibroblasts (15).

In another study performed by Joel Schlessinger et al. a cream with 8% of GlcNAc and 4% of triethyl citrate was developed. This cream was applied on the neck for a period of 16 weeks. The results showed that there was an increase in the production of hyaluronic acid and collagen and a reduction in the wrinkles of the neck (16).

1.2.2. Hyperpigmentation treatment

Hyperpigmentation of the skin, although not a disease, has a high psychological impact and can affect the quality of life. It occurs when there is an overproduction of melanin in some areas of the skin. It can appear e.g. after sun damage, acne or inflammation (17).

Some commercialised creams contain hydroquinone, a lightening agent, that it is responsible for various cases of skin irritation and contact dermatitis and ultimately was proved to be carcinogenic when used for a long time and in high concentrations.

An alternative to hydroquinone is GlcNAc. It can stop the production of melanin by inhibiting tyrosinase glycosylation. Furthermore, GlcNAc also changes the expression of some genes, like epidermal turnover genes and anti-oxidant genes, related to melanin production (1,17).

1.2.3. Skin hydration

Hyaluronic acid is a compound produced by fibroblasts and is a polyanion that can bind to water molecules. This ability of binding with water molecules allows it to control skin hydration and water transport (18).

As GlcNAc increases the number of fibroblasts and keratinocytes, it leads to a higher amount of hyaluronic acid in the upper epidermal layers and improves skin hydration. However, the results vary in several studies due to the variation in the formulation used as the vehicle. The formulations that lead to a higher increase in skin hydration were the formulations with liposomes (2).

1.2.4. Psoriasis

Psoriasis is characterised by the thickened and flaky skin that results from the hyperproliferation of keratinocytes and the production of inflammatory factors. (19)

In this phenomenon, one major inflammatory agent is involved: amphiregulin. In previous studies, amphiregulin was proved to be inhibited by heparin and sulphated polysaccharides. As glucosamine enhances the production of heparan sulphate by keratinocytes it can be used as an agent in the therapy of psoriasis (20).

1.2.5. Acne treatment

The anti-inflammatory activity of GlcNAc is also useful as an adjuvant in the treatment of acne as it decreases rapidly the number of lesions within two weeks, according to a study performed by Barbara A. et al. with an 8% GlcNAc gel. Compared to a 10% benzoyl peroxide cream, the GlcNAc gel induced a higher decrease in both inflammatory and non-inflammatory lesions within 4 weeks of treatment but was overcome after this period by the benzoyl peroxide cream. In the first 4 weeks GlcNAc was more efficient in reducing erythema, peeling and burning and was more well tolerated than benzoyl peroxide (21).

1.3. Safety of glucosamine

Glucosamine is safe in the diabetic population as it does not increase the glucose levels and it is not used as a caloric source (3,22).

According to Luyun Zou et al. “Glucosamine is metabolised via the hexosamine biosynthesis pathway leading to the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is a substrate for multiple glycosylation reactions catalysed by various GlcNAc transferases, including a unique *O*-linked GlcNAc (*O*-GlcNAc) transferase (OGT)” (9).

Thus, GlcNAc is incorporated in the molecules that need amino sugars to produce glycosaminoglycans (GAGs) that constitute the lubricant fluids and when linked to other proteins it forms the constituents of the skin, tissue and cartilage (23).

2. Skin Delivery

2.1. Skin physiology

The skin accounts for about 15% of the total body weight, which makes it the largest organ of the body. It plays a crucial role as it functions as a barrier against the external environment, either physical, chemical or biological and also plays a part in avoiding excessive loss of fluids and in thermoregulation (24).

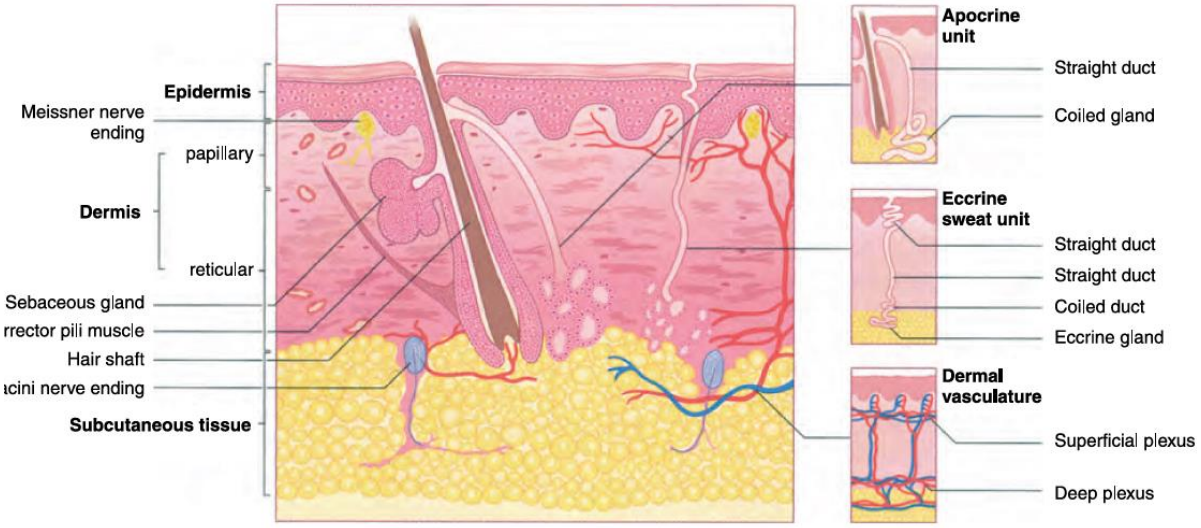


Figure 3 - Anatomical structure of human skin (24).

It has two main layers: epidermis and dermis as depicted in figure 3.

The epidermis is the most external layer, composed by keratinocytes (95%), melanocytes, Langerhans cells and Merkel cells (5%). It is divided in four major layers based on the differentiation stage of keratinocytes (25).

From the bottom, the epidermis is composed by the stratum basale, a single layer of keratinocytes that is in direct contact with the dermis, followed by the spinosum and granulosum strata and lastly the stratum corneum, the outermost layer.

The corneocytes in the stratum corneum represent the physical barrier, together with the cell-cell junctions, while the chemical barrier is composed by the lamellar lipid bilayers, hydrolytic enzymes and macrophages present in the several layers.

The stratum basale is responsible for the renovation of the skin with only 15% of the keratinocytes involved in this process as the others are in a quiescent state and will only activate when further proliferation (wounds or physical injury) requires so. The keratinocytes produce lipids and structural proteins, keratin being the major protein (26).

The differentiation of keratinocytes occurs with their migration through the four layers of the epidermis and is called keratinisation. In this process, the keratinocytes start in a synthetic phase, in the first three layers of the epidermis, and finish in a degradative phase where they lose their cellular organelles, after which they are called corneocytes, in the stratum corneum. This process takes at least 28 days, 14 days for the migration from the basal layer to the stratum corneum and 14 days for the migration from the stratum corneum to the skin surface (24).

In a normal state, there is a balance between the proliferation and the desquamation processes, but in skin disorders such as psoriasis the proliferation process is increased, resulting in a state of hyperproliferative hyperkeratosis with formation of parakeratotic scales (26). The apoptosis process is very important as it protects from mutated and damaged cells and its dysregulation can lead to tumours in the skin (24).

Other important cells present in the stratum basale are Langerhans cells and melanocytes. The Langerhans cells are responsible for the immunologic barrier of the skin and take part in the process of allergic contact dermatitis. Melanocytes are responsible for the production of melanosomes that contain melanin. The melanosomes protect the keratinocytes from ultraviolet radiation. The number and size of the melanocytes and the nature of melanin are some of the factors that determine the colour of the skin (25,26).

The stratum corneum is the outermost layer and is composed by corneocytes surrounded by lipids organised as lamellar bilayers, also known as the “brick and mortar” model, where the corneocytes are the bricks and the extracellular lipid matrix is the mortar. The lipid mass is synthetised by the keratinocytes and is composed of ceramides, cholesterol and free fatty acids. The ceramides, which are the major component of the lipid mass, are important for the organisation of the lamellar bilayer and cholesterol promotes the mixing of the different lipids, the flexibility of the lamellar bilayers and inhibits the enzymatic degradation of corneodesmosomes. The free fatty acids are mostly saturated, as are the ceramides. Any dysregulation in the composition of the lipid mass can compromise the integrity of the cutaneous barrier (27,28).

The main function of the stratum corneum is to protect the skin from excessive loss of water and electrolytes and prevent the entry of hydrophilic substances. The movement of water through the stratum corneum can be measured by the transepidermal water loss (TEWL), which increases in proportion with the damage level of the skin. Additional to the protective function of the lipids, the skin has an acidic pH, approximately 5, that protects against infections and dermatitises. Changes in the pH of the skin can be triggered by endogenous factors like

sweat and sebum or exogenous factors like cosmetics and topical antibiotics and lead to skin disorders such as dermatitis (26,29).

The other main layer of the skin is the dermis. The dermis represents 90% of the skin mass and is divided in the papillary layer, the outer part of the dermis, and the reticular layer, the inner part of the dermis. It is a layer composed by connective tissue, collagen and elastic fibres (30).

The dermis is extremely vascularised and contains sweat and sebaceous glands and hair follicles. The vasodilation and vasoconstriction of the vasculature are responsible for the thermal regulation. The main cells of the dermis are the fibroblasts, which are responsible for the production and renovation of the extracellular matrix, and the macrophages, that eliminate foreign material and damaged tissue (26).

Fibroblasts produce reticulum fibres, elastic fibres, and collagen. Collagen, which is the most prevalent component of the dermis, is a family of fibrous proteins and is also found in tendons, ligaments and the lining of bones. Although there are four types of collagen, the major one is type I. Collagen is responsible for the resistance of the skin against mechanical stress and the elastic fibres are responsible for the elasticity, but have a poor capacity to resist deformation (24). Collagen and elastic fibres are involved by a gelatinous substance, the ground substance, which is composed of glycosaminoglycans (GAGs) and proteoglycans (25).

The ground substance has multiple functions such as creating a unique environment that protects against viral infections, damaged immune cells and may be essential in mitosis.

One of the glycosaminoglycans present in the ground substance is hyaluronic acid which is a non-sulphated glycosaminoglycan and consists of repeated polymeric disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid linked by a glucuronic β bond schematically shown in figure 4 (31).

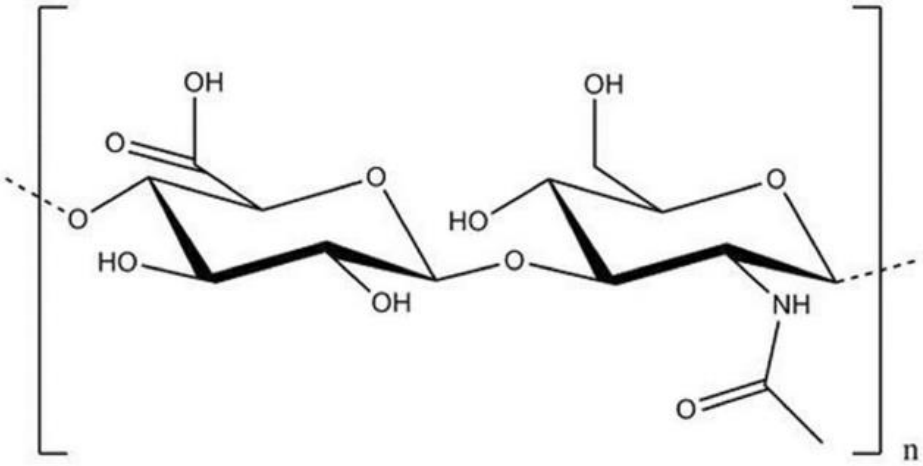


Figure 4 - Chemical structure of hyaluronic acid – bond between the D-glucuronic acid (on the left) and the N-acetyl glucosamine (on the right) (18).

Hyaluronic acid has several purposes such as tissue hydration, space filing, lubrication, cellular regulation and its production is increased in tissue repair and disease processes, controlling the activation of inflammatory cells (31).

2.2. Penetration routes

Although the skin functions as a barrier, several studies done so far prove that with the right compounds and formulations it is possible to deliver molecules across the stratum corneum down to the dermis. There are two different concepts: skin penetration and skin permeation. The first one refers to the passage of an ingredient into the skin for a local action, which means up to the epidermis, and the second one refers to the passage of an ingredient across the skin for a systemic action and for that it has to reach the dermis and the blood vessels that are in it (32).

In skin penetration there are two main penetration routes that a molecule can take when penetrating the skin, the transappendageal route and the transepidermal route illustrated in figure 5 (33).

The two routes of the transappendageal route are called shunt routes because compounds avoid penetration across the stratum corneum; they penetrate through sweat ducts and along the hair follicles with their associated sebaceous glands instead. These shunts are mostly utilised by small, charged molecules and large non-charged molecules (34).

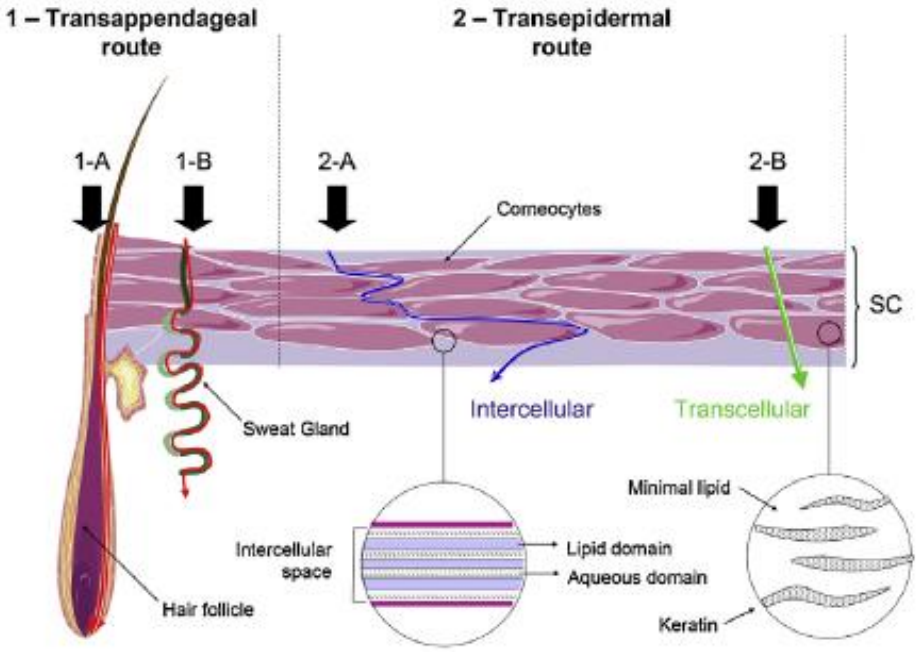


Figure 5 - Routes for penetration through human skin. 1 – Transappendageal route – shunt routes: A- hair follicle and B- sweat gland; 2 – Transepidermal route: A- intercellular pathway and B- transcellular pathway (33).

The other route is the transepidermal route and represents the full penetration across the stratum corneum and can be divided in a transcellular route and an intercellular route. The transepidermal route is the principal route for the majority of molecules and it occurs by passive diffusion (34,35).

In the intercellular pathway, the molecules pass between the corneocytes and only move in the extracellular lipid matrix. As this is a lipophilic domain, the main molecules of this route are lipophilic. On the other hand, in the transcellular pathway the molecules must cross several layers of corneocytes and extracellular matrix. As the interior of the corneocytes is hydrophilic and the extracellular matrix is lipophilic, the molecules have to partition and diffuse through both domains. The transcellular pathway is more often used by small hydrophilic molecules (27).

2.3. Factors that affect skin penetration

Skin penetration is influenced by the conditions of the skin and by the physical and chemical properties of the molecule and the formulation in which it is included.

Some of the skin variables affecting skin penetration are the age, site, temperature, pH, metabolism and state (hydration, disease). On the other hand, the penetration also depends on the penetrant, the vehicle and the interaction between the vehicle and the penetrant. Other factors that have an important part in the absorption are contact time and the frequency of application (36).

An important equation that summarises the percutaneous absorption is Fick's first law of diffusion,

$$J = -D \frac{dc}{dh} \qquad (1)$$

In which the diffusion flux, *J*, depends on the diffusion coefficient, *D*, the chemical potential of the penetrant, associated with the activity coefficient, the concentration of penetrant, *c*, and the thickness of the skin, *h.* This means that higher concentrations increase the penetrant's absorption and an increase in the thickness of the skin has the opposite effect (33).

Fick's first law can be simplified to,

$$J = k_p . \Delta c \qquad (2)$$

Where the Δc represents the concentration gradient across the skin (27). The k_p parameter represents the permeability coefficient and can be estimated by the Potts and Guy's equation, in which the *Koct* is the octanol water partition coefficient and the *MW* is the molecular weight of the penetrant,

$$\log k_p \ (cm\ s^{-1}) = -2.7 + 0.71 \log Koct - 0.0061\ MW \qquad (3)$$

This equation shows that molecules with a higher partition coefficient tend to have a higher flux but larger molecules will slow down the diffusion process (37).

Not only the mass of the molecule is important, but also its hydrophilicity. The molecules that penetrate more easily have sizes below 500 mDa and an octanol water partition coefficient between 1 and 3 (27).

Therefore, the main properties for skin absorption are the diffusion coefficient, that depends mostly on the shape and size of the molecule, the partition coefficient and the solubility, which is influenced by the penetrant's state of ionisation. (37,38) Other factors are the melting point, volatility, polarity, crystalline form and the ability to form hydrogen bonds (27,36).

2.4. Topical delivery

There are two concepts when using the skin as a delivery route: topical delivery and transdermal delivery.
Topical delivery is designed for a local effect and produces a low systemic concentration of the drug. (39) It is commonly used for skin disorders such as several infections and inflammations and is also used for prophylaxis like the use of sunscreen (40).

Topical dosage forms include creams, gels, lotions and ointments containing a high quantity of oleaginous substances that promote skin hydration by creating a barrier that restricts water loss, particularly in W/O emulsions. This is an important aspect as drugs are more likely to penetrate hydrated skin due to the several channels, intra- and intercellular, that it has, which leads to improved absorption. In some topical formulations (e.g. local anaesthetics) vasoconstrictors are included to minimise systemic delivery and extend the local action, as the example of epinephrine (40).

2.5. Transdermal delivery

Skin permeation is especially important in transdermal delivery systems as the rate and extension of absorption have a direct influence in the blood concentration of the drug as it has to be maintained at therapeutic levels during use (40).

An alternative to the oral route is the transdermal route and the first transdermal system was approved by FDA in 1979. This transdermal system was a three-day patch with systemic delivery of scopolamine. The recognition of the transdermal system became more significant with nicotine patches ten years later. There are three generations of transdermal systems (41).

The first generation accounts for the majority of the transdermal patches that are commonly constituted by lipophilic, small and potent drug molecules required in a low dose (28,41).
There are several types of transdermal patches such as single layer drug in adhesive, multi-layer drug in adhesive, vapour patch, reservoir system, matrix system and micro reservoir system (34).

The second generation of transdermal systems emerged with the use of enhancers as a way to promote skin penetration and consequently the amount of drug that enters the systemic circulation. The purpose of the enhancer is to improve the permeability through the stratum corneum by increasing the driving force for the molecule to penetrate the skin without damaging the tissues. Examples of these enhancers are chemical enhancers, non-cavitation ultrasounds and iontophoresis (41).

Chemical enhancers, which have lower costs, are the most researched as they can be included in topical formulations and might increase the solubility of the drug. The disruption of the lipid bilayer can be caused by the insertion of an amphipathic molecule or by the extraction of lipids in a nanometer scale (41,42).

Some of the chemical enhancing strategies are the use of liposomes, water, solvents, surfactants, emulsions, dendrimers and the use of prodrugs. The main disadvantage of chemical enhancers is their higher propensity in developing skin irritation even at low concentrations (41).

The third generation of transdermal delivery systems has its main focus on the stratum corneum and in increasing the disruption of the lipid bilayer without affecting the innermost tissues. In this generation, strategies such as combinations of chemical enhancers, biochemical enhancers, stripping, electroporation, microneedles, thermal ablation and microdermabrasion are used (28,41).

The combination of chemical enhancers reduces the skin irritation caused by the chemical enhancers used in the second generation and allows the delivery of larger molecules (41).

2.6. Considerations about topical and transdermal delivery

The skin as an alternative route to the oral route has several advantages. One of the advantages is that it is not influenced by the factors involved in the gastrointestinal system like the pH, the interactions with food, the first-pass metabolism in the liver and it can be used when the oral route is unavailable in cases such as vomits and diarrhoea. Also, it can be used with drugs with a narrow therapeutic index (34).

Topical formulations have a local action and are required in small doses, which makes them ideal for dermatologic conditions and their low systemic effects reduce the toxicity of the drug in naïve cells and reduce the side effects (43,44).
In some cases, as the application of topical formulations lasts longer, the frequency of administration is reduced, improving the compliance of the patient (28).

Transdermal delivery allows a stable drug concentration and since the patches are easy to apply and painless, it makes them a successful route in chronic treatments (40,45).
Transdermal delivery is also an alternative to the hypodermic injections and as it is a non-invasive technique that does not require needles, decreasing the possibility of infections and increasing the compliance by being less painful (41).
However, there are disadvantages associated with this route, mainly since the skin is a complex barrier. The drug has to be efficient in low doses, which limits the number of drugs available for these formulations, it has to have specific physicochemical properties such as low molecular mass and has to be more lipophilic, which once more limits the drugs that can be used, especially hydrophilic molecules, macromolecules and peptides. Topical and transdermal formulations are quite often associated with skin irritation, partly owing to the enhancers and other excipients used (34,41).
The most common side effects of topical delivery are allergic reactions and erythema. Other challenges of this route are the chemical challenges due to the pH dependent stability and photosensitivity of some compounds (44).

3. Modulation of skin penetration

3.1. Penetration enhancers

There are four types of penetration enhancers that can be used to increase the penetration of topical drugs: chemical enhancers, vesicular systems, novel drug delivery systems and enzymes (32).

Chemical enhancers are the most widely used penetration enhancers for transdermal delivery. The main mechanism of action is the disruption of the lipid bilayer, either by increasing the fluidity of the membrane or by opening pores. Examples of chemical enhancers are isopropyl myristate, isopropyl alcohol, glycerol and propylene glycol (32).

Another form of penetration enhancers are vesicular systems. These systems are composed of amphipathic molecules and, in this way, they can mix with the skin lipids (ceramides and cholesterol) or penetrate the skin through the lipid-water interface. In various studies, liposomes composed of phospholipids were proved to have better penetration results compared to liposomes composed of lipids imitating the skin lipids. Liposomes disrupt the lipid bilayer and increase the absorption of drugs (32).

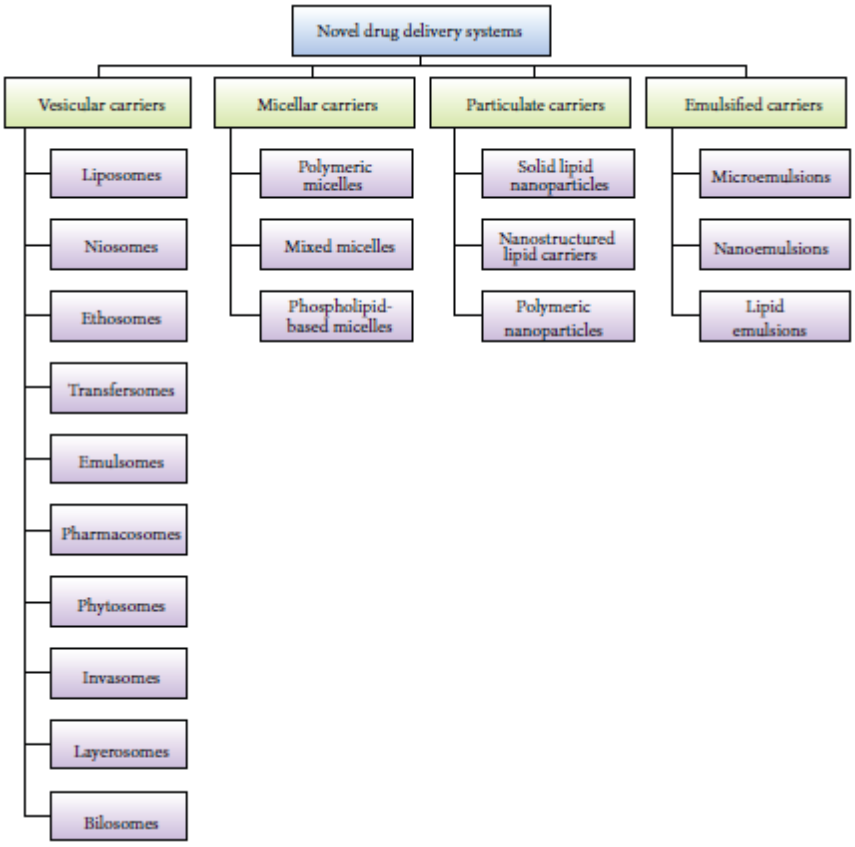


Figure 6 - Examples of the “Novel Drug Delivery Systems” (43).

There are many types of carriers that penetrate the stratum corneum according to their size. The bigger carriers, like liposomes and microemulsions, integrate into the lipid bilayer due to their lipidic surroundings. Flexible membrane vesicules and flexible carriers like ethosomes

(vesicules with a high content of alcohol) use the intercellular pathway, passing between the corneocytes and moving in the extracellular lipid matrix. The smaller carriers such as lipid nanoparticules and nanoemulsions can penetrate either by the intercellular pathway or the transcellular pathway (44).

These carrier systems and others are part of the “Novel Drug Delivery Systems” (NDDS) and have several advantages such as transportation and protection of the active ingredient, biocompatibility and increase the number of drugs that can be administered through topical delivery (44). Examples of NDDS are shown in figure 6.

Another type of penetration enhancers are the enzymes. Enzymes can react with the enzymes that are already present in the skin, for example, inhibiting the synthesis of fatty acids or cholesterol and allowing the penetration of the molecule. Furthermore, enzymes can be applied on the top of the skin and induce hydrolysis of the corneocytes and create bonds between intracellular proteins (32).

3.2. Formulating for efficacy

A concept introduced by Wiechers et al, 2004 is “Formulating for efficacy” (FFE) in which the right solvent or solvents of the formulation are chosen to improve the penetration of the active ingredient in the stratum corneum (35).

For a greater penetration of the active ingredient, three main factors must be considered, the octanol/water partition coefficient, the diffusivity and the stratum corneum/formulation coefficient. The first two factors depend only on the molecule of the active ingredient. The molecule must have a high octanol/water partition coefficient, which can be increased by changing the chemical structure of the molecule and it has to have a high diffusivity, which can be increased by adding hydroxyl groups to the molecule so that it can form hydrogen bonds with the skin. The third factor is the stratum corneum/formulation coefficient which depends on the polarity of the formulation. The first two factors are more difficult to change so the goal of the Formulating for Efficacy is to find the right polarity and the right concentration of every ingredient in the formulation (35).

A novel concept is the Relative Polarity Index in which the stratum corneum is seen as a solvent and its polarity was determined to be 0.8. The polarity of the stratum corneum and the polarity of the molecule of the active ingredient are known and placed on a vertical logarithmic line that starts with high values of polarity and ends with high values of lipophilicity at the bottom (35).
The polarities of the active ingredient, the skin and formulation can be estimated through the Hansen Solubility Parameter (HSP). (46) The HSP values are calculated considering three components of the molecule: the components that refer to the van der Waals bonds and the hydrogen bonds and the polar component. The closer the values of HSP are, the greater is their solubility in each other (38).

Three scenarios are possible: the polarity of the active ingredient is the same as the stratum corneum (which is less common), the polarity of the active ingredient is higher than the stratum corneum or the polarity of the active ingredient is less polar than the stratum corneum (35).
The difference between the polarity of the molecule of the active ingredient and the polarity of the stratum corneum is called “Penetrant Polarity Gap” (PPG) and the difference is absolute so that the value is always positive. This difference is then applied on the scale from the polarity

of the active ingredient either to more polar values or more lipophilic values and the polarity that the formulation should have is set. In order to have a higher concentration of the penetrant in the stratum corneum, the difference between the polarity of the active ingredient and the formulation must be as large as possible. It creates a driving force for the active ingredient to move to the stratum corneum as it has a polarity closer to stratum corneum. This is explained by the fact that “likes dissolves like” (35,46).

As the solubility in the skin increases, the diffusion coefficient increases and the penetration speed is also increased (38).

However, this means that the active ingredient is less soluble in the formulation. According to Wiechers et al, 2004 the polarity of the formulation should be calculated:

Polarity of formulation > polarity of penetrant + penetrant polarity gap
Polarity of formulation < polarity of penetrant – penetrant polarity gap

Using the Relative Polarity Index the formulation needs to have a certain polarity that can be achieved by mixing two solvents or by a solvent with the right polarity. Usually the first situation is better as the first solvent is used to dissolve the active ingredient and guarantee a high concentration of the penetrant in the formulation and the second solvent is used to decrease the solubility of the active ingredient and increase the penetration driving force (35).

4. Work plan

In the market, there are several supplements of glucosamine, all intended for oral use. According to Dalifardouei et al 2016,

“Oral GlcN pharmacokinetics data including absorption, distribution, metabolism and elimination (ADME) indicates it has a low and erratic oral bioavailability which is truly unclear in humans” (2).

Although the absorption through the oral route is high, the concentration of GlcNAc in the plasma is much lower than the concentration obtained through the intramuscular or intravascular routes, with only 26% of the dose being absorbed (22,47).

Though the half-life of glucosamine is short, it has a rapid distribution in the liver, kidneys and articular cartilages (22).

Even though that less than 1% of the original dose reaches the articular cartilages it has a high potency and demonstrates great results in the treatment over time in osteoarthritis (23).

The purpose of this study is to develop a topical formulation of GlcNAc, as it is a metabolically active prodrug of glucosamine and is more stable for topical formulations than glucosamine (23,48). The aim is to increase the amount of glucosamine at the site of action and use it in several therapeutics due to its pharmacological properties.

Other characteristics that make GlcNAc a good candidate for transdermal delivery are the low molecular mass, its satisfactory solubility in lipids, high potency in low doses and being actively transported to the articular cartilage via the glucose transporter system (10,23). Besides, it has a low price and a high availability in the market (23).

5. Objectives

- Research about previous formulations of topical GlcNAc and their concentrations;
- Collect the UV spectra of GlcNAc;
- Collect the UV spectra of possible solvents to verify if they do not absorb at the same wavelength as GlcNAc;
- Test the DNS method for GlcNAc quantification in topical formulations;
- Selection of solvents based on their ability to dissolve GlcNAc;
- Formulating gels with different solvents and ratios;
- *In vitro* release studies with Tuffryn® and Strat-M® membranes to select the best sample.

6. Materials

GlcNAc, 95% plus purity and glucose were purchased from Sigma-Aldrich. Propylene glycol and glycerol were purchased from SciChem (UK). Paraffin liquid was purchased from Vickers Laboratories (UK). Dipropylene glycol was obtained from Aromatic Flavours & Fragrances Europe (UK).

The DNS reagent, potassium sodium tartrate tetrahydrate, the phosphate buffered saline tablets and sodium hydroxide were purchased from Sigma-Aldrich (Germany). Oleic acid was obtained from Benchmark (UK). The Carbopol® Ultrez 10 was purchased from Lubrizol (EUA). Purified water was obtained with the Synergy® Water Purification System from Merck Milipore.

7. Methods

An exhaustive work was done by consulting the sources PubMed and the University of the Arts London Library. The research used terms such as *glucosamine*, *n-acetyl glucosamine*, *quantification methods* and *formulating for efficacy*. According to the Formulating for Efficacy concept the following solvents were selected: glycerol, propylene glycol, caprylic/capric triglycerides (CCT), dipropylene glycol, liquid paraffin, isopropyl myristate (IPM) and oleic acid. For the quantification of GlcNAc the following method was adapted: DNS method.

7.1. Pre-formulation studies

7.1.1. Selection of the excipients

UV spectrum scanning

To select the right compounds for the topical formulation, spectroscopy assays were performed with GlcNAc in hydrophobic and hydrophilic solvents.

The hydrophilic solvents were glycerol and propylene glycol and the hydrophobic solvents were caprylic/capric triglycerides (CCT), dipropylene glycol, liquid paraffin, isopropyl myristate (IPM) and oleic acid.

The spectra for GlcNAc and all the solvents was collected by using the Jenway 7315 Spectrophotometer. The absorbance of each compound was checked over a wide range of

wavelengths, i.e. within the capacity of the equipment (200 to 800 nm). The wavelength and the absorbance at which the highest signal (peak) was recorded was identified as the maximum wavelength, λ_{max}.

Solubility Tests

For the solubility tests, standard calibration curves for each of the solvents were performed.

Since the hydrophobic solvents failed to dissolve the GlcNAc, the solubility tests were only performed with water, glycerol and propylene glycol. GlcNAc was dissolved in each of the solvents and the absorbance was measured for each concentration of GlcNAc. These tests were done in triplicate for each of the solvents and the saturation of solutions was performed overnight, in constant magnetic stirring at 600 rpm without heating. To obtain only precipitate-free samples solvents, the solutions were centrifuged at 2000 rpm for 10 min in the Centurion Scientific Limited PrO-Analytical C2006. The supernatant was collected and the absorbance was measured in the spectrophotometer. As the concentrations were too high for the spectrophotometer to measure it was necessary to dilute the solutions for each of the solvents and consider the dilution factor in the calibration curves.

7.1.2. UV method for the determination of GlcNAc

Colourimetric method

The method chosen to quantify GlcNAc was the DNS-Colourimetric method, an efficient and quite advantageous method for detection of reducing sugars. This method is based on the oxidation of the carbonyl group of the sugar and the subsequent reaction with an UV/Vis absorbing reagent under alkaline conditions. This reagent promotes the reduction of 3,5 – dinitro salicylic acid to 3-amino-5-nitrosalicylic acid, an aromatic compound with a maximum absorption at 545 nm, in the red area of the visible spectrum. The DNS reagent is prepared by mixing 0.160 g of sodium hydroxide in 2 mL of ultra-pure water. Afterwards, 0.10 g of 3,5-dinitrosalicylic acid and 5 mL of ultra-pure water are added. After complete dissolution, 3 g of potassium tartrate are added and the volume is topped up to 10 mL with ultra-pure water.

Briefly, 250 µL of reagent (3,5-dinitrosalicylic acid, DNS) were added to 2 mL of purified water and 250 µL of glucose 0,1% or GlcNAc 0,1% in a falcon tube. All the excipients were tested in the same conditions substituting the sugar (GlcNAc or glucose) with the excipients to test if the excipients had a reaction with the DNS reagent. The falcon tubes were heated at 95 °C in a water bath for 5 min to promote the colourimetric reaction. Then, after cooling down for 15 min at room temperature, 2.5 mL of each sample were measured by UV/Vis spectrophotometry at two wavelengths (480 and 540 nm).

7.2. Formulation studies

Formulations development

Five gels of 50 g with 2% GlcNAc were prepared for the permeation tests (Table 1). The carbomer used for all the gels was 0.5% Carbopol® Ultrez 10 polymer. To formulate the gels, the carbomer was dispersed in half of the water necessary and homogenised with an IKA Labortechnik RW 16 basic machine at speed 7. After complete homogenisation, the GlcNAc was dissolved in the rest of the water and added to the formulation as well as the rest of the

excipients, according to each formulation. When all was mixed, the pH was corrected to 6 with Sodium Hydroxide 20% solution, using a Metrohm® 914 pH/conductometer, as this is the pH that the carbomer chains expand to give the gel texture to the formulation.

Table 1 - Qualitative and quantitative composition (% w/w) of the gels.

	F1	*F2*	*F3*	*F4*	*F5*
GlcNAc	2	2	2	2	2
Carbomer	0.5	0.5	0.5	0.5	0.5
Water	97.5	48.75	32.5	65	32.5
Glycerol	------	48.75	65	32.5	32.5
Propylene glycol	------	------	-----	-----	32.5

In Vitro Permeation of GlcNAc with Tuffryn® and Strat-M® membranes

The five formulations were tested *in vitro*. These tests were performed using vertical glass Franz diffusion cells (Vertical Diffusion Cell Test System Model HDT 1000 from Copley Scientific™). The receptor solution was Phosphate-Buffered Saline (PBS) pH 7.4 which easily dissolves GlcNAc, besides having a similar physiological composition of the blood. The receptor volume was ≈7 mL. The membrane used to mimic the skin was the HT Tuffryn® Polysulphone Membrane Disc Filters 25 mm.

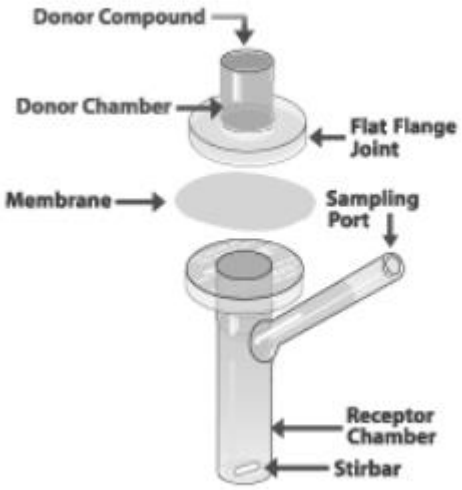


Figure 7 - Franz cell experimental set up (49).

These membranes were soaked 30 min before the *in vitro* test in Phosphate-Buffered Saline (PBS) pH 7.4. The shiny side of the Tuffryn® membrane was facing upwards in the lid of the Franz cell to stay in contact with the formulation in the donor chamber. Afterwards, the Franz cell was assembled as shown in figure 7 (49). Parafilm™ was put around the lid to prevent the evaporation of the phosphate buffer caused by heating. Before covering the sampling port, all the bubbles were removed. Then, the formulation (0.1 g) was applied onto the donor chamber

and covered with more Parafilm™ to prevent the evaporation of the formulation's components. The amount of formulation in each Franz cell and the time of its assemble were recorded. The cells were kept at 32 ºC and stirring at 50 rpm during the entire process. The samples were collected from the sampling port at standard times: 0.5, 1, 2, 3 and 4 h. After collecting each sample, the phosphate buffer volume was restored to maintain sink conditions.

Each formulation was tested with ten Franz cells.

This process was repeated with the formulations that showed the best results, but with Strat-M® Membranes 25 mm and with three Franz cells per formulation.

8. Results

UV spectrum scanning

As the spectra of CCT and the oleic acid were too similar to the spectra of GlcNAc, these two solvents were discarded from the formulation and the rest of the solvents proceeded to the solubility tests.

Solubility Tests

The solubility tests were only performed with the excipients that had the best results in the spectra collection. Since the hydrophobic excipients were not able to dissolve GlcNAc, they were discarded from the solubility tests. The wavelength chosen to measure the concentration of GlcNAc in each solvent was the wavelength of the peak characteristic of the solvent. The results for water, propylene glycol and glycerol are shown below:

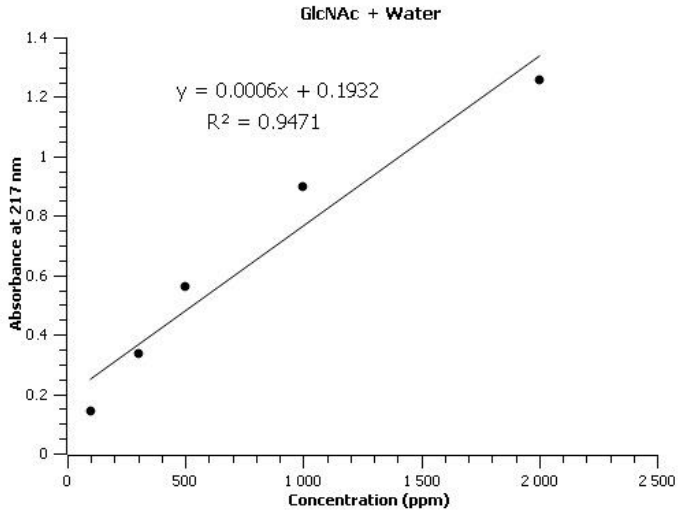


Figure 8 - Calibration curve of GlcNAc in water.

Based on the calibration curve and the absorbance of the saturated solution, the concentration of GlcNAc in the saturated solution of water was 206 × 10^3 ppm (Figure 8).

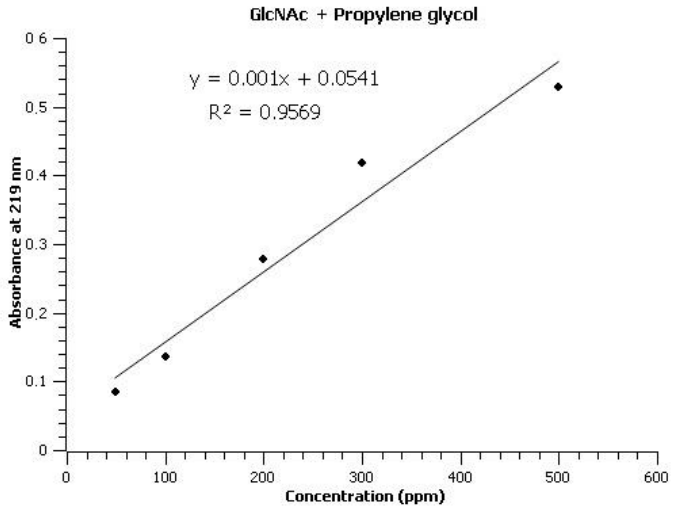


Figure 9 - Calibration curve of GlcNAc in propylene glycol.

For the propylene glycol, the concentration of GlcNAc in the saturated solution was 7.39×10^3 ppm (figure 9).

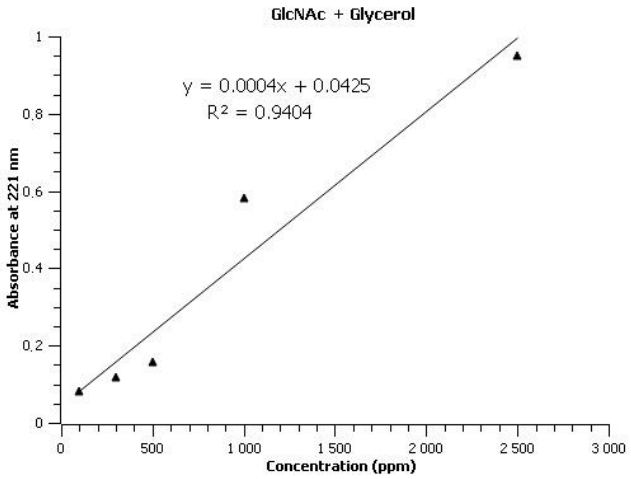


Figure 10 - Calibration curve of GlcNAc in glycerol.

Based on the calibration curve and the absorbance of the saturated solution, the concentration of GlcNAc in the saturated solution of glycerol was 40.38×10^3 ppm (figure 10).

Colourimetric method

GlcNAc 0.1%, glucose 0.1% and all the samples were tested with the DNS method. The GlcNAc and glucose samples were yellow and after heating they became red. The excipients remained in a light orange. The method described was analysed and the spectrophotometer indicated that the samples were too dark to read. The samples were diluted with ultra-pure water to become less dark but the colour turned to yellow and the wavelength shifted to smaller values. As it could be a variation in the pH solutions that changed the colours, the samples were diluted with a buffer, a sodium hydroxide and potassium tartrate solution, to maintain the pH 13 that is the pH of the reaction, but the colour still changed to yellow.

After several experiments changing the amounts of the DNS reagent, sample and buffer, the DNS method was abandoned. A possible suggestion would be to use microtiter wells to measure the concentration of the samples as they require lower amounts of sample. Therefore, the sample would not get so dark and the equipment would be able to read it without diluting the sample. Another possibility would be to work with an equipment able to read more concentrated samples.

After obtaining a calibration curve of GlcNAc in Phosphate-Buffered Saline (PBS) pH 7.4 with the NanoDrop™ 2000 Spectrophotometer from Termo Scientific at 202 nm (figure 11) the concentration of GlcNAc began to be measured by direct UV spectrophotometry.

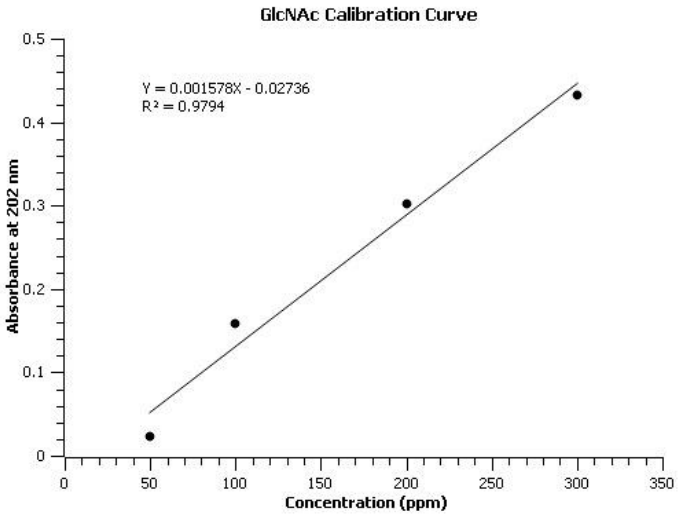


Figure 11 - Calibration curve of GlcNAc in PBS.

The readings were made at two wavelengths (202 and 220 nm) and the difference of the absorbance obtained between the two readings was applied in the calibration curve. The spectra obtained for the formulation of GlcNAc in water at 0.5 h with the Tuffryn® membranes is shown below (Figure 12). Appendix I has all the spectra taken from 0.5 h to 4h for the five formulations used in the present study with Tuffryn® and Strat-M® membranes.

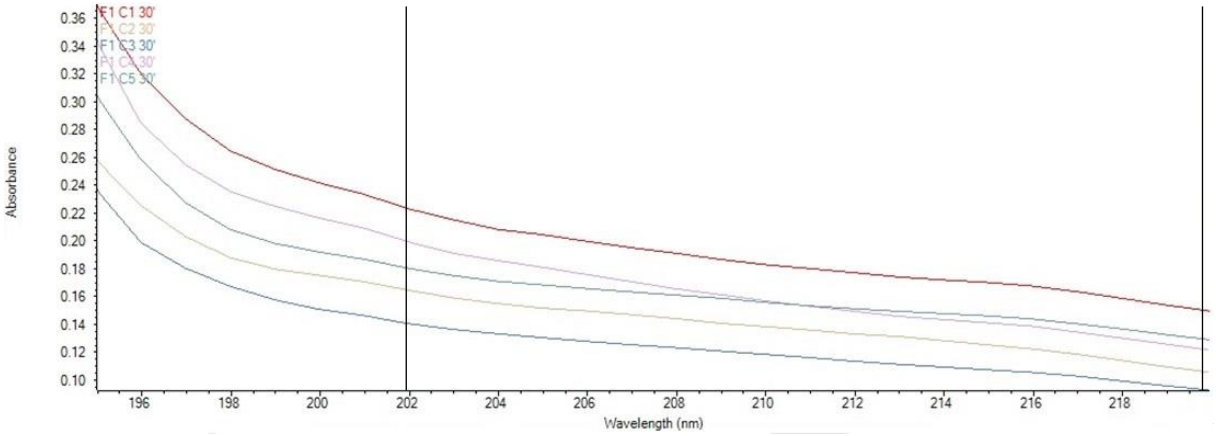


Figure 12 - Spectra of GlcNAc in water at 0.5 h with the Tuffryn® membrane.

Figure 12 schematically shows the location of the measurement points used to obtain the amount permeated during the experiment. Figure 13 and Figure 14 summarise the results for the Tuffryn® and the Strat-M® membranes.

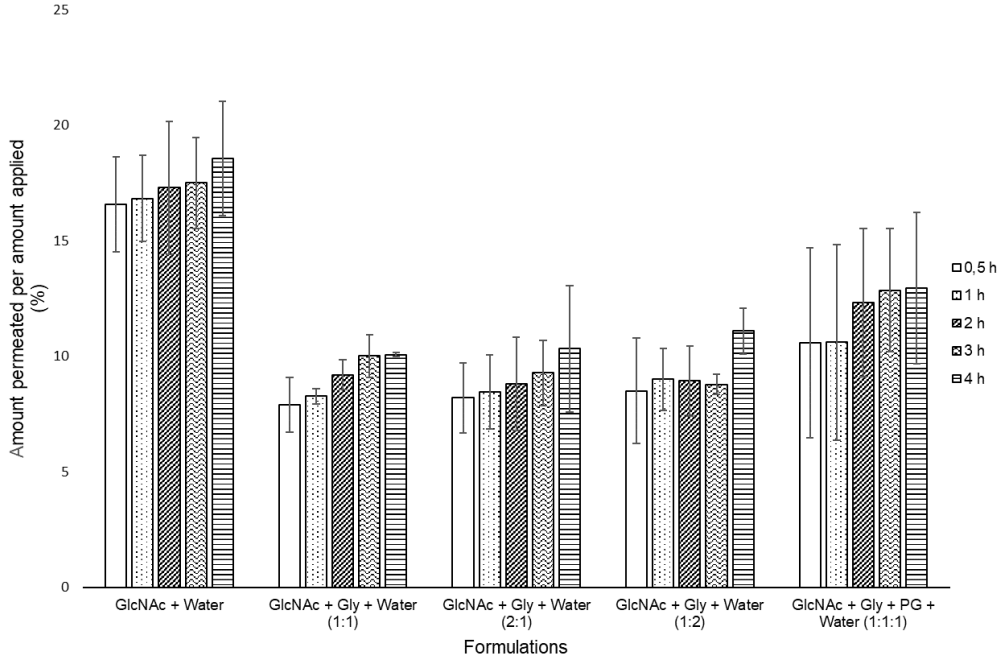
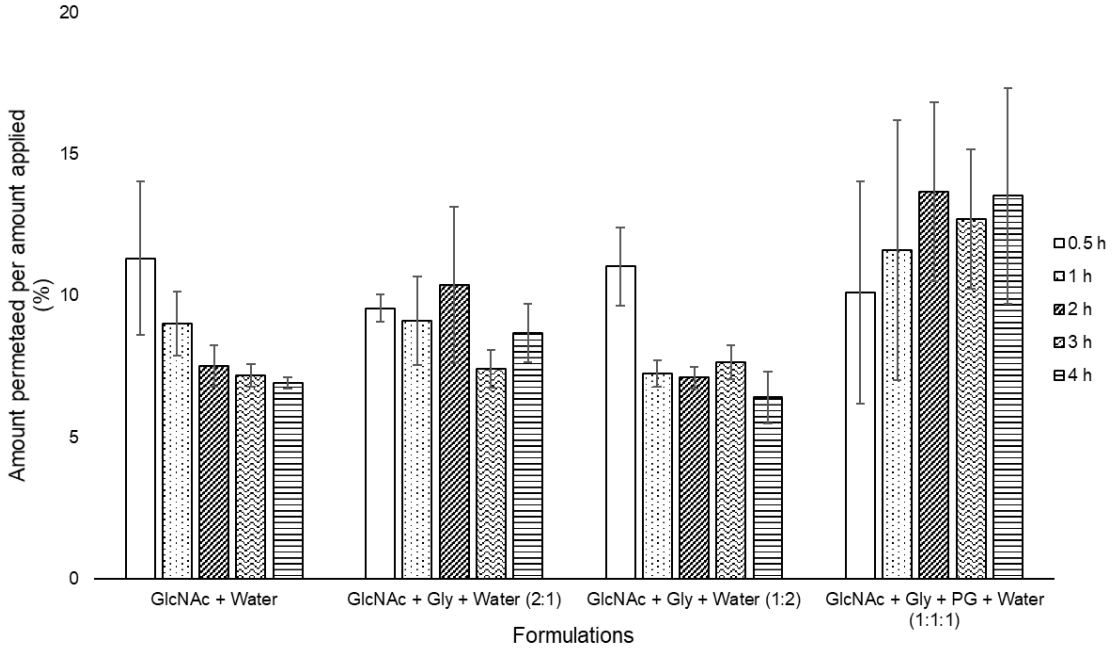


Figure 13 - *In Vitro* permeation of GlcNAc with the Tuffryn® membranes.

The graph above shows that the formulation of GlcNAc in water is the one that released GlcNAc faster and in higher quantities reaching almost 20% at the end of the four hours, followed by the formulation of GlcNAc in glycerol, propylene glycol and water. The formulations of GlcNAc with different ratios of glycerol have similar results, albeit slightly lower than the formulation with the mixture of glycerol, propylene glycol and water (1:1:1) with half of the amount permeated in GlcNAc with water.



. **Figure 14** - *In Vitro* permeation of GlcNAc with the Strat-M® membranes.

The results with the Strat-M® membrane show a striking contrast with the results obtained with the Tuffryn® membrane. For the formulation of GlcNAc in water, the amount of GlcNAc permeated with the Strat-M membrane decreased with time, while it increased with the Tuffryn® membrane. For the formulations with glycerol, different trends can be seen for the amount permeated:

- GlcNAc in glycerol and water (2:1) slightly decreased over time;
- GlcNAc in glycerol and water (1:2) decreased over time;
- GlcNAc in glycerol, propylene glycol and water (1:1:1) increased over time.

The formulation of GlcNAc in glycerol, propylene glycol and water was the one with the largest amount of GlcNAc permeated and the only one where permeation increased over the time. In the rest of the formulations, the permeation of GlcNAc tended to decrease as the test continued.

9. Discussion

The spectra obtained with the formulations was not precise. Therefore, the absorbance of the GlcNAc released from the formulations was determined by subtracting the absorbance of the peak with the absorbance of the baseline. The wavelength of the peak was defined at 202 nm as it was the peak observed in the spectrum of GlcNAc in water. The wavelength of the baseline was settled at 220 nm since at this wavelength there was no other noise or solvent peaks. This was the way around the problem to get accurate results as the method was applied in all the samples. The shifting of the baseline could be due to the instrument itself or changes in the environment like vibration, temperature and changes in the voltage.

The *in vitro* tests were performed with the Tuffryn® membranes and the Strat-M® membranes (Figure 15). These two membranes intend to imitate the skin. The Tuffryn® membrane is constituted by a single layer of hyprophilic polysulphone, which is extremely low protein binding, and has a total thickness of 145 µm (50). On the other hand, the Strat-M® membrane is more complex and is closer to the human skin as it is constituted by two layers of polyethersulphone (PES) on top of one layer of polyolefin, with a total thickness of 300 µm. The polyethersulphone (PES) layer is similar to the polysulphone layer of the Tuffryn® membrane, but with lower protein retention and higher resistance to diffusion (51).

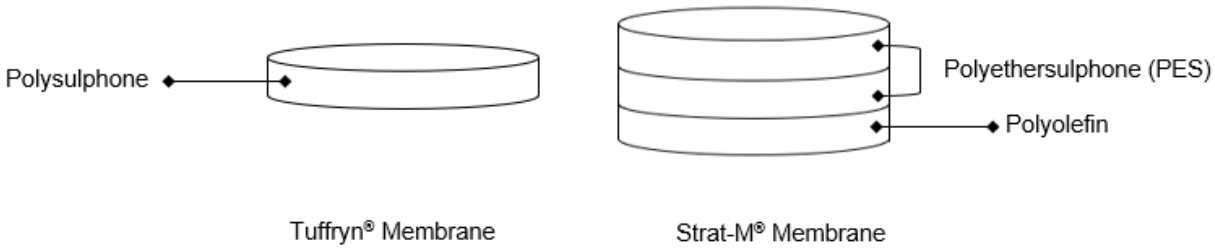


Figure 15 - Scheme of the composition of Tuffryn® and Strat-M® membranes abridge from (50,51).

The first measurement was taken at 0.5 h after the start of the experiment. The results are summarised below in Figure 16, which also indicates typical values for the amount permeated from bibliography.

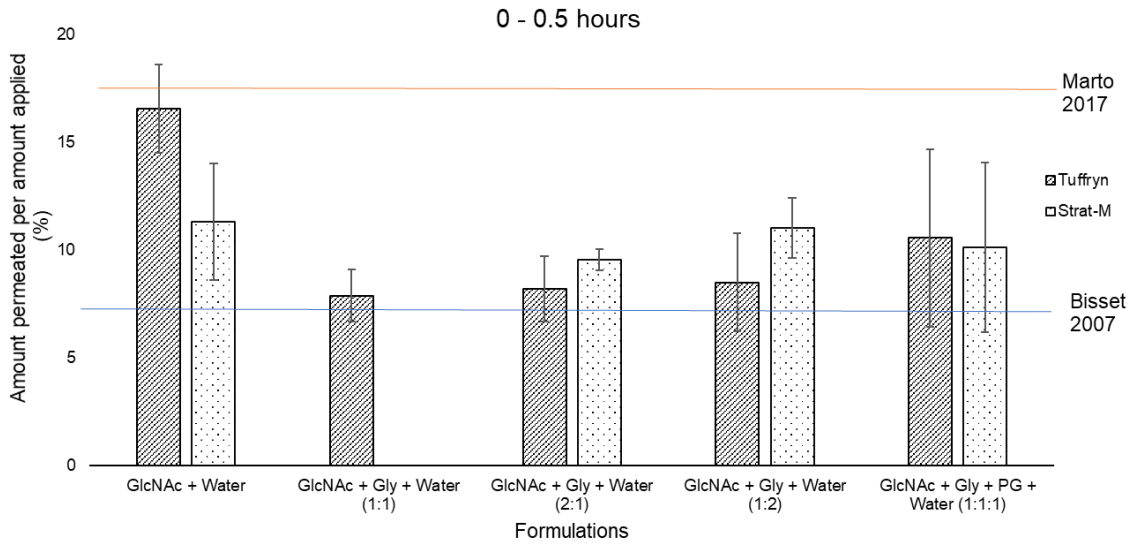


Figure 16 - Comparison of the results with the Tuffryn® and the Strat-M® membranes in the first half hour of the *in vitro* test with the bibliographic results. The formulation of GlcNAc in water and glycerol (1:1) was not tested with the Strat-M® membrane.

It can be seen that all values obtained in the first half hour of the experiments are within the values referred in bibliography, which confirms the good quality of the data measured in the present experiment (48,52). Some additional observations can be made:

- In the formulation of GlcNAc in water the amount of GlcNAc permeated in the Tuffryn® membrane was the highest of all experiments and in the Strat-M® membrane the second highest, though in the latter case it was very close to the formulations with chemical enhancers. GlcNAc in water is quite free to move directly to the membrane and to start permeating through, and as the Tuffryn® membrane is a thinner, more porous membrane compared with Strat-M®, it was able to permeate at a higher rate in the beginning of the experiment.
- On the other hand, GlcNAc with chemical enhancers may be adsorbed at the layers of Polysulphone and Polyethersulphone in the Tuffryn® and Strat-M® membranes respectively, consequently taking more time to diffuse from the layers to the receptor chamber. This phenomenon explains why in all formulations of GlcNAc with chemical enhancers, only about half of GlcNAc permeated within the same time, compared with GlcNAc in water through the Tuffryn® membrane.
- For the formulations with chemical enhancers, the amount permeated through both membranes was similar. This showed the potential effectiveness of glycerol and propylene glycol in promoting the permeation through the membranes, specifically the Strat-M® membrane, as these substances create a high enough driving force for GlcNAc to diffuse through such a restrictive membrane.

Figure 17 summarises the entire data measured between 0.5 h and 4 h for Tuffryn® and Strat-M® membranes.

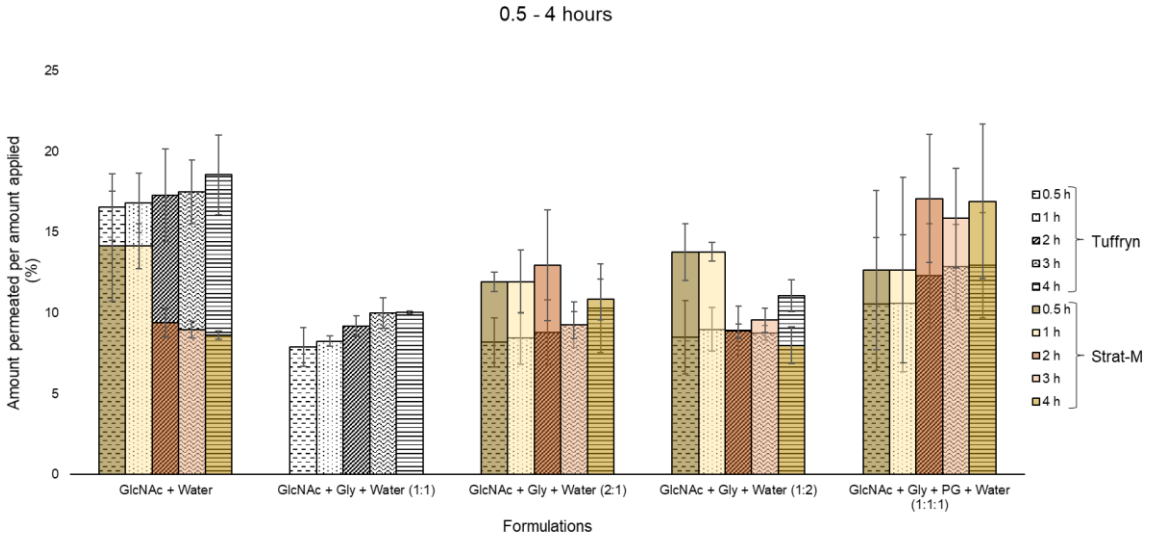


Figure 17 - Comparison of the results with the Tuffryn® and the Strat-M® membranes between the first half hour of the test and the fourth and last hour of the test. The formulation of GlcNAc in water and glycerol (1:1) was not tested with the Strat-M® membrane.

Generally comparing the results obtained with the Tuffryn® and Strat-M® membranes, it can be seen that in the formulation of GlcNAc in water there was a substantial decrease in the amount of GlcNAc permeated from Tuffryn® to Strat-M® membrane. This was expected as Strat-M® membrane is a more restricting membrane and in this formulation GlcNAc had no help from any chemical enhancer to permeate through the membranes. Looking at the formulations with chemical enhancers, the amount permeated was relatively similar in both membranes. This proved that chemical enhancers are effective in promoting permeation through the more restrictive Strat-M® membrane.

Calculation of the k_p value

Permeation of drugs is influenced by their permeability coefficient (k_p). The formula elaborated by Potts and Guy (equation 3) in page 22 allows to determine this coefficient of GlcNAc, as it can be calculated through its molecular weight and its octanol/water partition coefficient.

Table 2 - Chemical parameters of GlcNAc. (1,17)

Parameter	
Molecular weight	221.21 g/mol
Log $K_{oct/water}$	- 3.3
k_p	$4{,}06 \times 10^{-7}$ cm s^{-1}

The theoretical value of k_p of GlcNAc indicates the speed by which the molecule diffuses through the stratum corneum. It does not indicate the amount permeated (35). By nature, due to its high hydrophilicity and molecular weight, GlcNAc should present a low speed of permeation. Hence, why poor permeation occurred in the formulation of GlcNAc in water for the Strat-M® membrane, while higher values were obtained for the formulations of GlcNAc with chemical enhancers.

Application of the Higuchi model

As the release of GlcNAc is not linear, it does not follow a zero-order model. Therefore, a possible model that can describe the permeation of GlcNAc through the membranes is the Higuchi model. The Higuchi model, developed by Higuchi in 1961, is one of the models that evaluates the permeation of a topically applied drug from a matrix, with the membrane functioning like a reservoir. This model is based on the formula,

$$M_t^{gel} = k_H t^{1/2} \qquad (4)$$

where M_t^{gel} is the concentration of a drug in a matrix at a certain square root of time and k_H is the dissolution constant. This model assumes a higher concentration of the drug in the matrix and a unidirectional diffusion in sink conditions (53). The dissolution constant k_H and the correlation coefficient R^2 in Table 3 and Table 4 were obtained from applying a regression line to the results in Figure 17 for the intervals 0 – 0.5 h, 0.5 – 4 h and 0 – 4 h for the Tuffryn® and the Strat-M® membranes.

Table 3 - Higuchi method applied to 0 – 0.5 h; 0.5 – 4 h and 0 – 4 h in the formulations tested with the Tuffryn® membrane.

	0 - 0.5 h		**0.5 – 4 h**		**0 - 4h**	
	k_H	R^2	k_H	R^2	k_H	R^2
GlcNAc + Water	23.42	1	1.39	0.89	11.47	0.50
GlcNAc + Glycerol + Water (1:1)	14.94	1	1.85	0.97	7.97	0.66
GlcNAc + Glycerol + Water (2:1)	12.03	1	1.50	0.89	6.19	0.60
GlcNAc + Glycerol + Water (1:2)	11.58	1	1.41	0.51	6.13	0.69
GlcNAc + Glycerol + Propylene glycol + Water (1:1:1)	11.17	1	2.16	0.92	6.04	0.63

Table 4 - Higuchi method applied to 0 – 0.5 h; 0.5 – 4 h and 0 – 4 h in the formulations tested with the Strat-M membrane.

	0 - 0.5 h		**0.5 – 4 h**		**0 - 4h**	
	k_H	R^2	k_H	R^2	k_H	R^2
GlcNAc + Water	16.01	1	-3.23	0.87	5.13	-0.15
GlcNAc + Glycerol + Water (2:1)	13.51	1	-1.01	0.23	5.78	0.16
GlcNAc + Glycerol + Water (1:2)	15.6	1	-2.62	0.58	4.87	-0.15
GlcNAc + Glycerol + Propylene glycol + Water (1:1:1)	14.31	1	2.43	0.74	8.31	0.67

Figure 18 and Figure 19 show the regression line applied to the 0.5 – 4 h interval for the Tuffryn® and the Strat-M® membranes, respectively, from where the values in Table 3 and Table 4 are determined. The regression lines for the 0 – 0.5 h and 0 – 4 h intervals are in Appendix II. The results in Table 3 and Table 4 show that the Higuchi model cannot properly describe the permeation of GlcNAc through the membranes for the entirety of the experiment, i.e. for the interval 0 – 4 h. However, if the experiment for both membranes is separated at 0.5 h and the Higuchi model is applied to the results before and after, for the intervals 0 – 0.5 h and 0.5 – 4 h, the correlation coefficient shows that the Higuchi model can describe the permeation behavior of GlcNAc through the membranes reasonably well. This indicates there was a phenomenon that started at 0.5 h which changed the permeation behavior of GlcNAc.

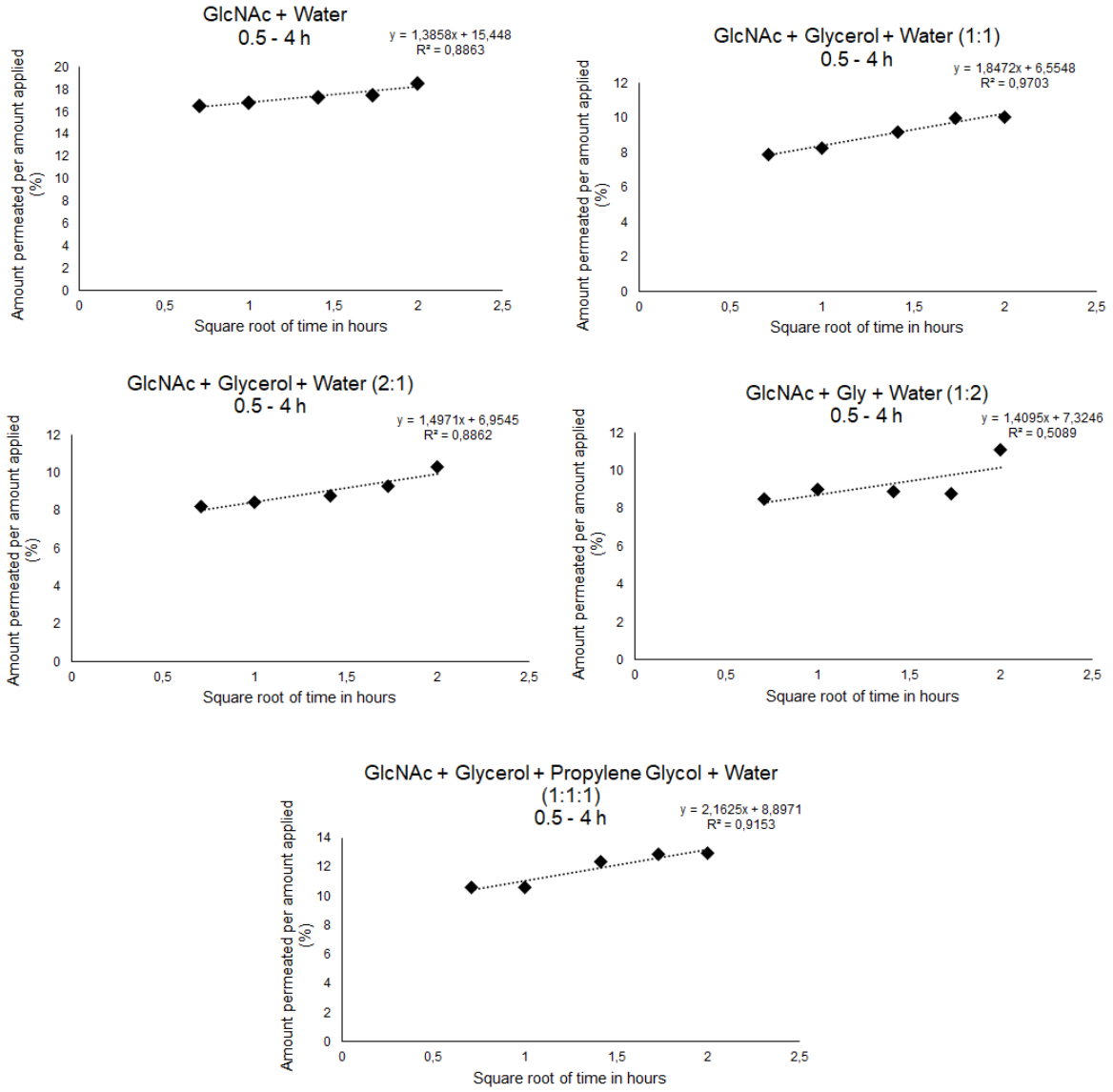


Figure 18 - Application of the Higuchi model in all the formulations tested with the Tuffryn® membrane for the interval 0.5 – 4 h and their respective dissolution constants.

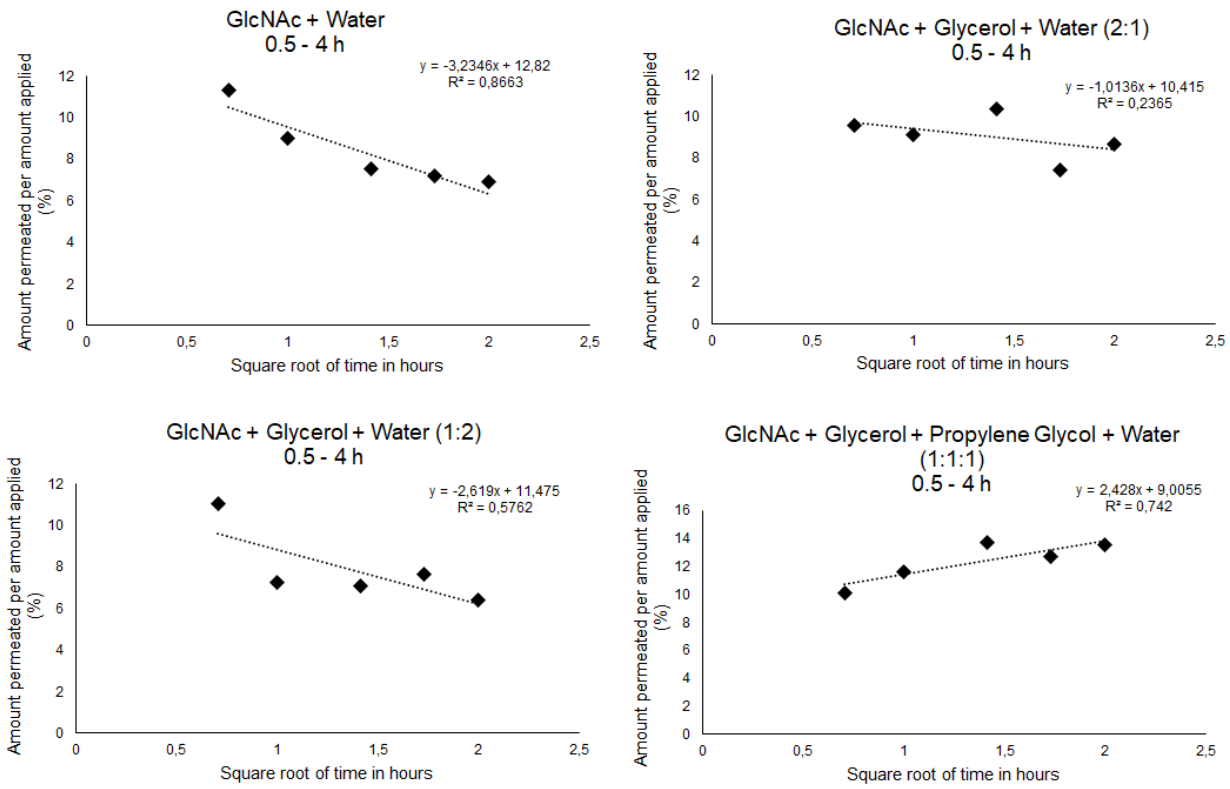


Figure 19 - Application of the Higuchi model in all the formulations tested with the Strat-M® membrane and their respective dissolution constants.

The slope taken from these graphs gives the dissolution constant of the Higuchi model. The values for the dissolution constant are summarised below.

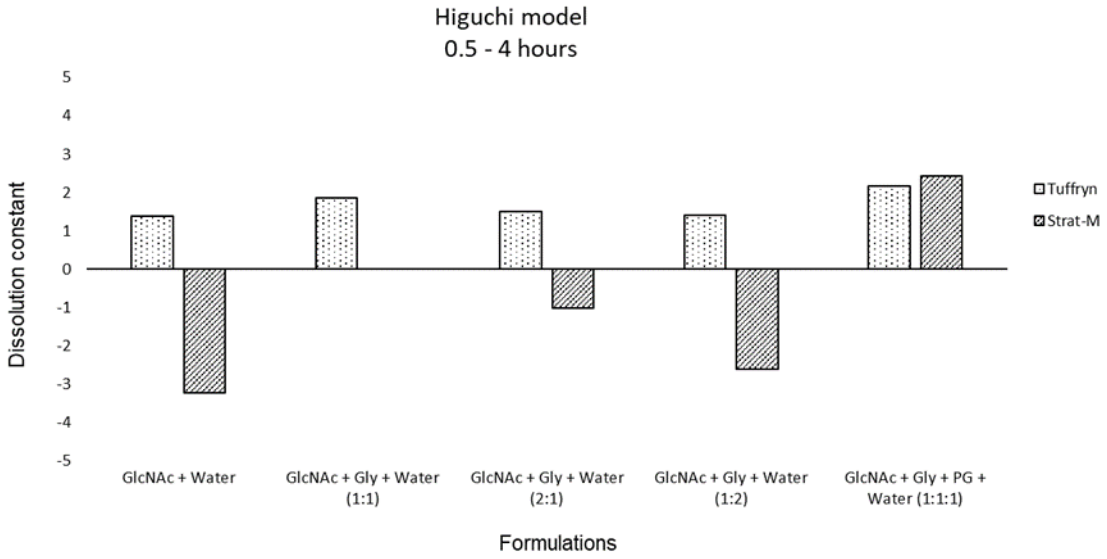


Figure 20 - Dissolution Higuchi constants for each formulation in Tuffryn® and Strat-M® membranes.

Analysing the dissolution constants for the different formulations some conclusions can be drawn:

- For the Tuffryn® membrane the dissolution constants were very similar.
- For the Strat-M® membrane it was observed that the dissolution constant was negative in almost all formulations. A negative value indicates that the concentration decreased with time.
- The formulation of glycerol, propylene glycol and water (1:1:1) was the only one with a positive result obtained for the dissolution constant in the Strat-M® membrane. It confirmed the positive results already obtained with the Tuffryn® membrane for this formulation.

The formulations of GlcNAc with chemical enhancers all showed a modest amount of GlcNAc permeated from 0.5 h to 4 h, indicated by negative or slightly positive dissolution constants. The mixture of GlcNAc, glycerol, propylene glycol and water showed the best results with the Strat-M® membrane, surpassing the results obtained with the Tuffryn® membrane. On the other hand, the formulation of GlcNAc in water showed a major decrease in the amount of GlcNAc permeated from the Tuffryn® membrane to the Strat-M® membrane, such that GlcNAc in water in the Strat-M® membrane presented the lowest dissolution constant of the entire experiment. This suggests that the combination of glycerol and propylene glycol, out of all formulations, had the optimal polarity to create a driving force for the permeation of GlcNAc.

According to the literature, a formulation with a good permeability through the skin should have a dissolution constant of approximately 40 (52). Results in Table 3 and Table 4 show that until 0.5 h of the experiment, the dissolution constant had values between half and one third of that. However, for the remaining of the experiment, even for the glycerol, propylene glycol and water (1:1:1) formulation, the dissolution constant was less than 10% of that value, and in some cases the dissolution constant was negative.

A possible reason for a negative dissolution constant could be an interaction of GlcNAc or glycerol with the components of the membrane. As polysulphone is used as a filtration method to separate GlcNac particles, the polysulphone cannot react with GlcNAc. (54) There might be a slight of or lack of interaction of glycerol and propylene glycol with polysulphone, according to the “Polysulphone Chemical Compatibility Chart” (55). Hence, neither GlcNAc nor the solvents should have significantly interacted with the membrane, which does not justify a negative dissolution constant.

Another possible factor contributing to the negative dissolution constant could be the fact that the amount of sample placed in each Franz cell was 0.1 g/cm^3 while the specifications are that 1 g/cm^3 should be applied. This means the amount of sample applied was 10 times lower than what was supposed. Furthermore, the sample of each Franz cell was taken from the arm of the Franz cell and not from the middle as it was assumed that the stirring kept the solution homogenous. Nevertheless, the proper way to collect the sample is from the middle of the receptor chamber, since stirring might not be enough to maintain homogeneity in the entire cell (56).

The most likely reason, however, could have been the crystallisation of GlcNAc in the membrane. This effect would explain the deceleration in the permeation of GlcNAc and the reason why the application of the Higuchi model to the results of the present experiment had to be separated at 0.5 h, as this may have been the time when crystallisation started to occur. The crystallisation of GlcNAc probably occurred due to the evaporation of water and

the fast permeation of the chemical enhancers through the membrane, leaving only GlcNAc in the membrane (33).

For a topical formulation to be effective, the active – in this case GlcNAc – needs to be delivered into the membrane with the highest thermodynamic activity as possible, so that it can be delivered to the deeper layers. According to the Formulating for Efficacy theory, this means that the active needs to be close to saturation and therefore subject to the possibility of crystallisation. The chemical enhancers used in the present study – propylene glycol and glycerol – are colloidal carriers that are absorbed by the stractum corneum, releasing GlcNAc by diffusion but as they permeate rapidly, they leave the active substance in the membrane, which induces its crystallisation due to the absence of solvent.

When GlcNAc started to crystallise, it did not pass from the donor chamber to the receptor chamber. The GlcNAc that was released in the first half hour was diluted after the collection of the first sample and the replacement with buffer to maintain the sink conditions, so the concentration of GlcNAc may have decreased with time, which could explain the negative dissolution constant. As depicted in Figure 20 this was the case for the majority of the formulations tested using the Strat-M® membrane. This effect was not so pronounced in the Tuffryn® membranes because they have less restrictive diffusion pathways compared to Strat-M®, therefore, in Tuffryn® membranes GlcNAc was still able to keep permeating slightly during the time of the experiment, causing a positive dissolution constant in the formulations without the propylene glycol. Only the formulation with GlcNAc, glycerol, propylene glycol and water showed a positive dissolution constant for both membranes, even though less than 10% of amount of sample required for a good permeability was used.

In addition, by looking at the theoretical value of k_p for GlcNAc, it can be concluded that this substance has a low natural permeability. So, it is possible that the use of chemical enhancers might not be the most effective way to deliver GlcNAc through the skin.

A probable alternative consists in nanostructuring GlcNAc to increase its permeability through both membranes and avoid its crystallisation in them. Nanostructured GlcNAc behaves as a small carrier and can permeate through the skin by the intercellular spaces and by the transcellular pathway.

10. Conclusion

Glucosamine is well known for its use as an oral supplement in the treatment of osteoarthritis. Recent studies proved the use of glucosamine not only as a therapeutic agent but also as a cosmetic agent. It can be used especially as an anti-ageing agent, due to the increase in the production of hyaluronic acid by fibroblasts, and it can be used in the hyperpigmentation as an alternative to hydroquinone. With its wide potential, novel formulations of glucosamine, particularly in the form of N-acetyl glucosamine, have appeared in the market. This work aimed to develop a topical formulation of GlcNAc that could deliver a higher amount of glucosamine through the skin and decrease the time of the treatment.

In this study, permeability tests were performed with Tuffryn® and Strat-M® membranes and the following conclusions can be drawn.

1. GlcNAc has a low permeability coefficient (k_p) which means a low rate of permeation. Therefore, the accelerators glycerol and propylene glycol were tested in different ratios for both membranes;
2. Only the formulation with a mixture of glycerol and propylene glycol increased the permeation in both membranes as it created the best vehicle polarity of all the formulations and, in this way, created a favourable driving force for the permeation of GlcNAc;
3. The measured values for all formulations showed a permeation potency of GlcNAc that was one order of magnitude lower than the value of 40 necessary for a good permeability, according to the bibliography. This is possibly due to a crystallisation effect caused by the evaporation of water and the fast permeation of the chemical enhancers through the membrane.
4. This crystallisation effect means it is possible that the usage of chemical enhancers might not be the most effective way to deliver GlcNAc through the skin.
5. A probable alternative consists in nanostructuring GlcNAc, so that it can permeate through the skin through the intercellular spaces and the transcellular pathway.

This work confirmed the potential of GlcNAc to be used in topical formulations, but further studies need to be performed in order to optimise the delivery of GlcNAc through the skin.

Below, some topics are proposed for future work:

- To test the formulations in a more controlled environment to prevent the obtaining of irregular spectra;
- To improve the understanding of the crystallisation mechanism using *in situ* and *ex situ* methods such as X-ray diffusion, fluorescence spectroscopy and infrared spectroscopy;
- To use different chemical enhancers or to incorporate GlcNAc in novel drug delivery systems to increase its permeation, avoiding the crystallisation effect and increasing the number of possible penetration routes;
- To use nanostructured GlcNAc and compare them with other penetration enhancers;
- To develop formulations based in other forms of glucosamine – glucosamine hydrochloride and glucosamine sulfate – with several chemical enhancers to test their permeability and exploit the therapeutic differences between the three forms of glucosamine supplements.

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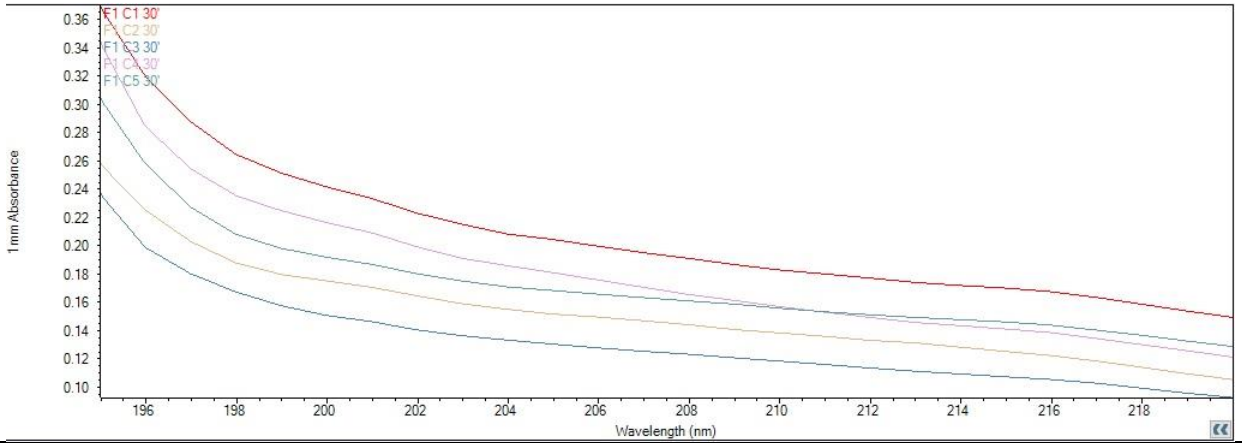
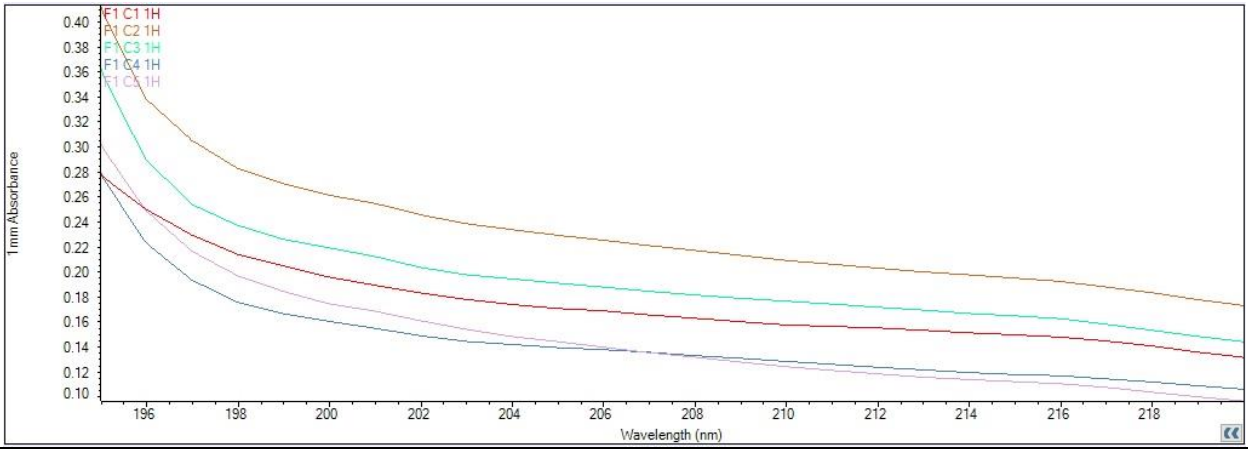
12. Appendix I

Tuffryn® Membrane – GlcNAc + Water

After 0.5h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11161	0.10964	0.10987	0.10067	0.10911
$m_{a,max}$	0.002324	0.002283	0.002283	0.002097	0.002272
A_{bl} (220 nm)	0.149	0.105	0.092	0.121	0.128
$A_{max,a,t}$ (202 nm)	0.223	0.164	0.14	0.199	0.18
$c_{a,t}$	64.23	54.73	47.76	66.77	50.29
$m_{a,t}$	449.61	383.11	334.32	467.39	352.03
$m_{a,t}/m_{a,max}$	19.346	16.781	14.612	22.289	15.494
Spectra					

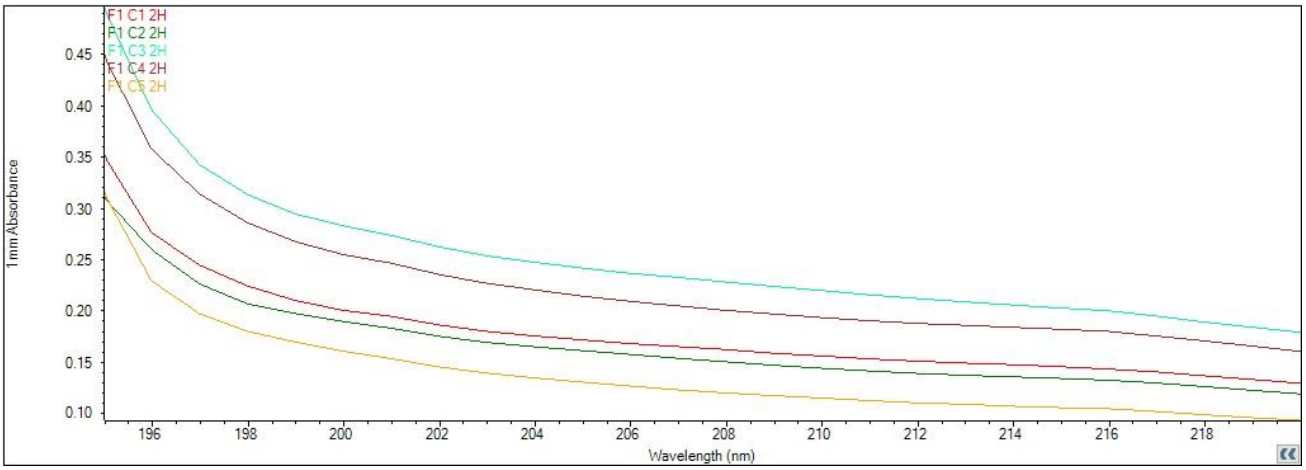


After 1h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11161	0.10964	0.10987	0.10067	0.10911
$m_{a,max}$	0.002324	0.002283	0.002288	0.002097	0.002272
A_{bl} (220 nm)	0.131	0.173	0.143	0.105	0.096
$A_{max,a,t}$ (202 nm)	0.183	0.245	0.203	0.148	0.160
$c_{a,t}$	50.29	62.97	55.36	44.59	57.90
$m_{a,t}$	352.03	440.79	387.52	312.13	405.30
$m_{a,t}/m_{a,max}$	15.148	19.308	16.937	14.885	17.839
Spectra					



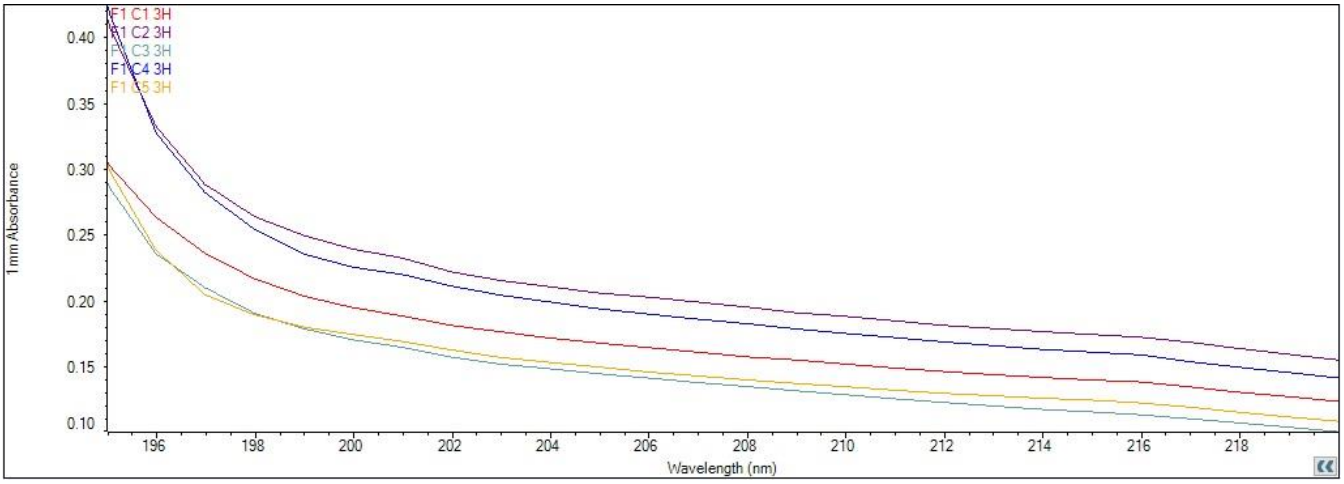
After 2h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11161	0.10964	0.10987	0.10067	0.10911
$m_{a,max}$	0.002324	0.002283	0.002288	0.002097	0.002272
A_{bl} (220 nm)	0.129	0.118	0.178	0.16	0.092
$A_{max,a,t}$ (202 nm)	0.185	0.174	0.262	0.235	0.144
$c_{a,t}$	52.83	52.83	70.57	64.87	50.29
$m_{a,t}$	369.81	369.81	493.99	454.09	352.03
$m_{a,t}/m_{a,max}$	15.913	16.198	21.591	21.654	15.494

Spectra



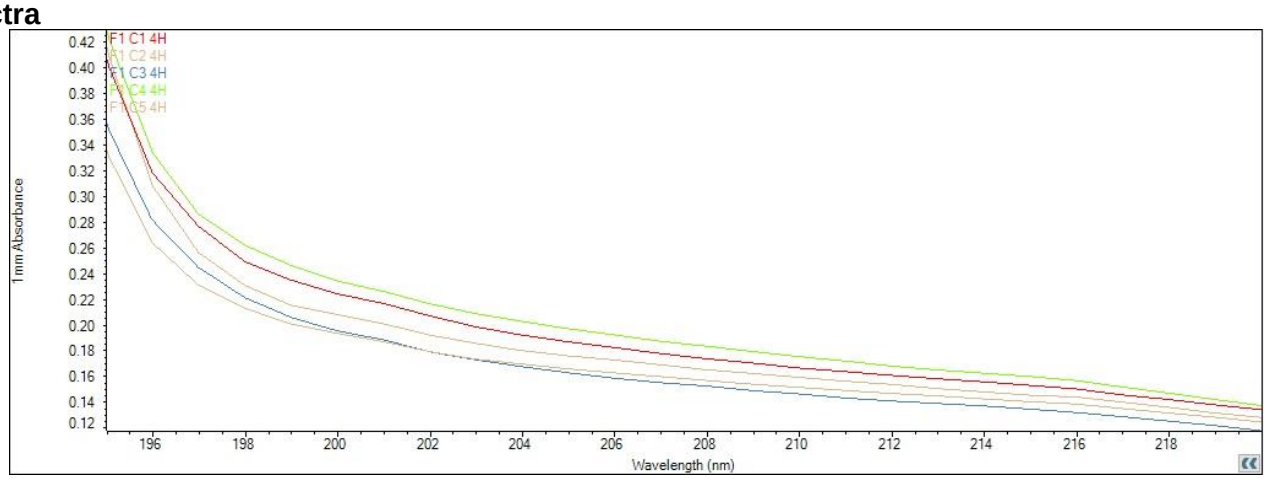
After 3h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11161	0.10964	0.10987	0.10067	0.10911
$m_{a,max}$	0.002324	0.002283	0.002288	0.002097	0.002272
A_{bl} (220 nm)	0.123	0.154	0.1	0.141	0.108
$A_{max,a,t}$ (202 nm)	0.181	0.221	0.157	0.211	0.162
$c_{a,t}$	54.09	59.80	53.46	61.70	51.56
$m_{a,t}$	378.63	418.60	374.22	431.90	360.92
$m_{a,t}/m_{a,max}$	16.292	18.336	16.356	20.596	15.886

Spectra



After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11161	0.10964	0.10987	0.10067	0.10911
$\mathbf{m}_{a,max}$	0.002324	0.002283	0.002288	0.002097	0.002272
$\mathbf{A}_{bl}$ (220 nm)	0.134	0.127	0.117	0.137	0.124
$\mathbf{A}_{max,a,t}$ (202 nm)	0.207	0.192	0.178	0.216	0.179
$\mathbf{c}_{a,t}$	63.60	58.53	56.00	67.40	52.19
$\mathbf{m}_{a,t}$	445.20	409.71	391.93	471.80	365.33
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	19.157	17.946	17.130	22.499	16.080

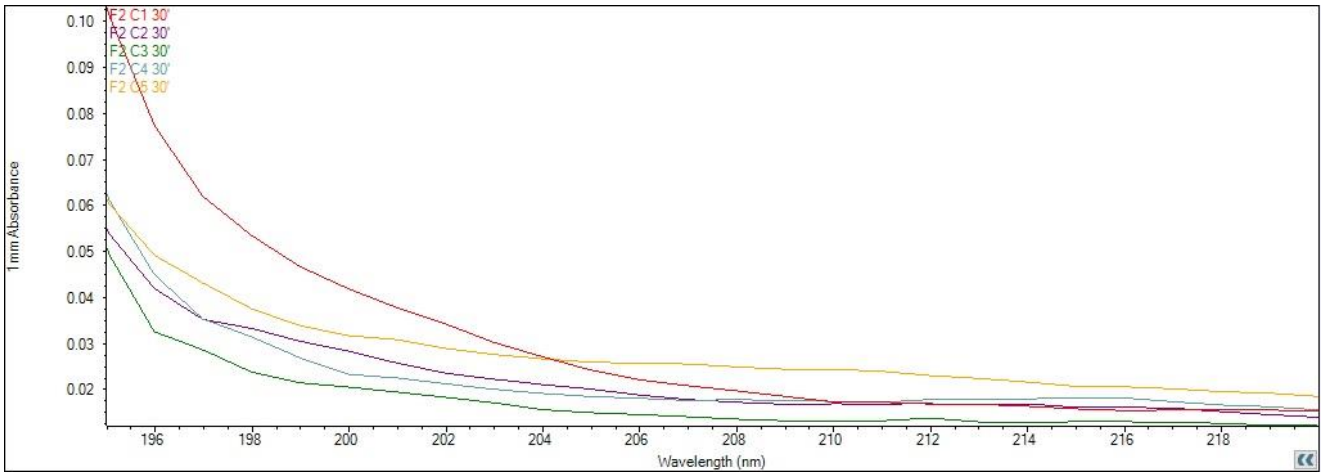
Spectra



Tuffryn® Membrane – GlcNAc + Glycerol + Water (1:1)

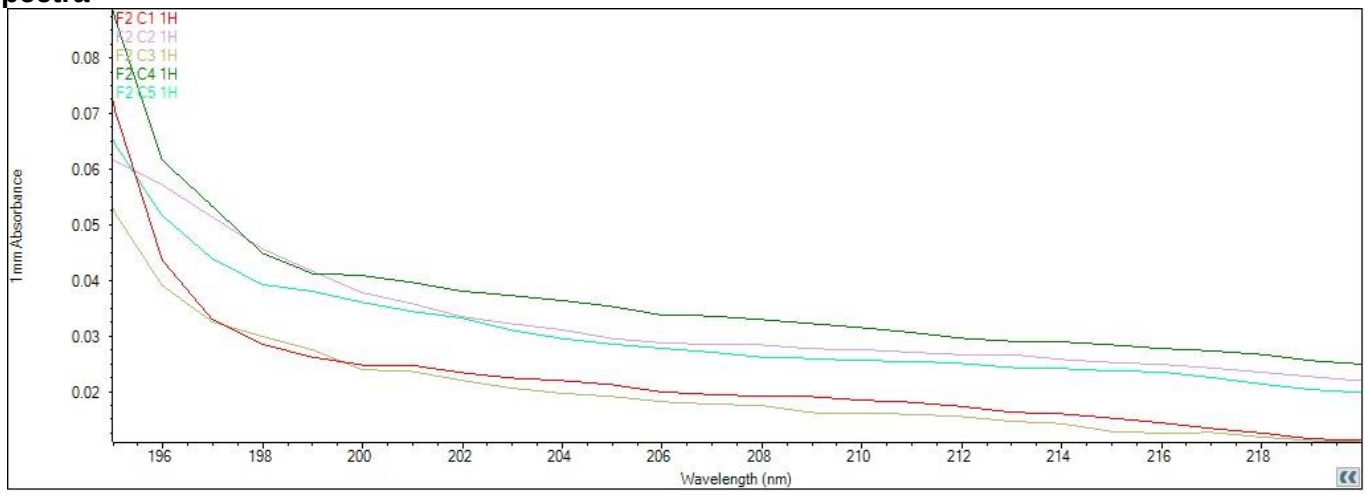
After 0.5h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.10542	0.10037	0.10949	0.10644	0.10356
$\mathbf{m}_{a,max}$	0.002120	0.002019	0.002202	0.002141	0.002083
$\mathbf{A}_{bl}$ (220 nm)	0.015	0.014	0.012	0.015	0.018
$\mathbf{A}_{max,a,t}$ (202 nm)	0.034	0.023	0.018	0.021	0.029
$\mathbf{c}_{a,t}$	29.38	23.04	21.14	21.14	24.31
$\mathbf{m}_{a,t}$	205.66	161.28	147.98	147.98	170.17
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	9.701	7.988	6.720	6.912	8.170

Spectra



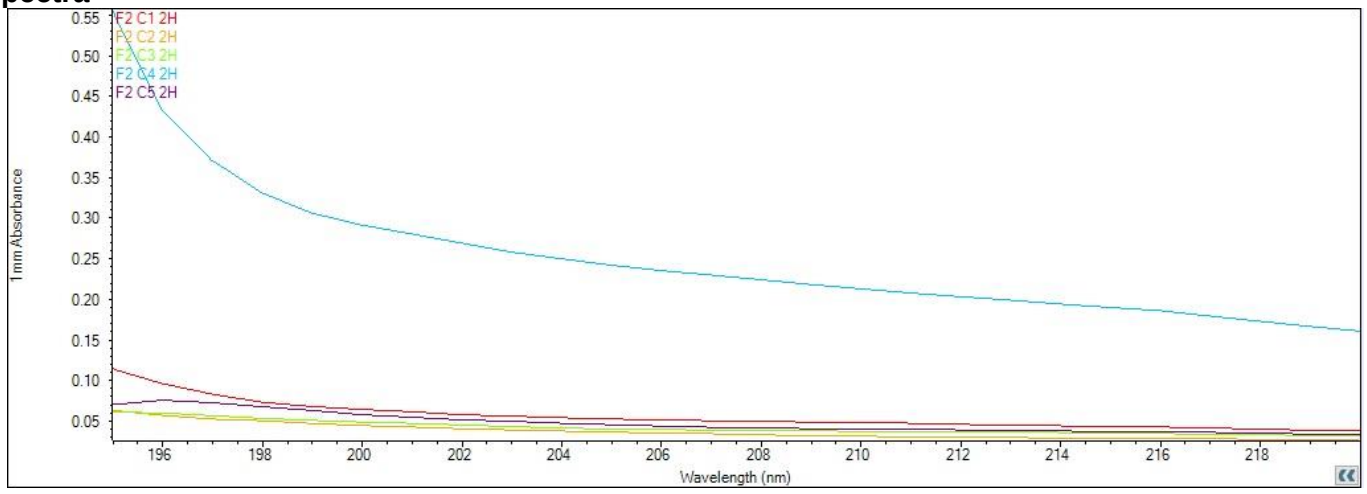
After 1h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.10542	0.10037	0.10949	0.10644	0.10356
$\mathbf{m}_{a,max}$	0.002120	0.002019	0.002202	0.002141	0.002083
$\mathbf{A}_{bl}$ (220 nm)	0.011	0.022	0.011	0.025	0.02
$\mathbf{A}_{max,a,t}$ (202 nm)	0.023	0.033	0.022	0.038	0.033
$\mathbf{c}_{a,t}$	24.94	24.31	24.31	25.58	25.58
$\mathbf{m}_{a,t}$	174.58	170.17	170.17	179.06	179.06
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	8.235	8.428	7.728	8.363	8.596

Spectra



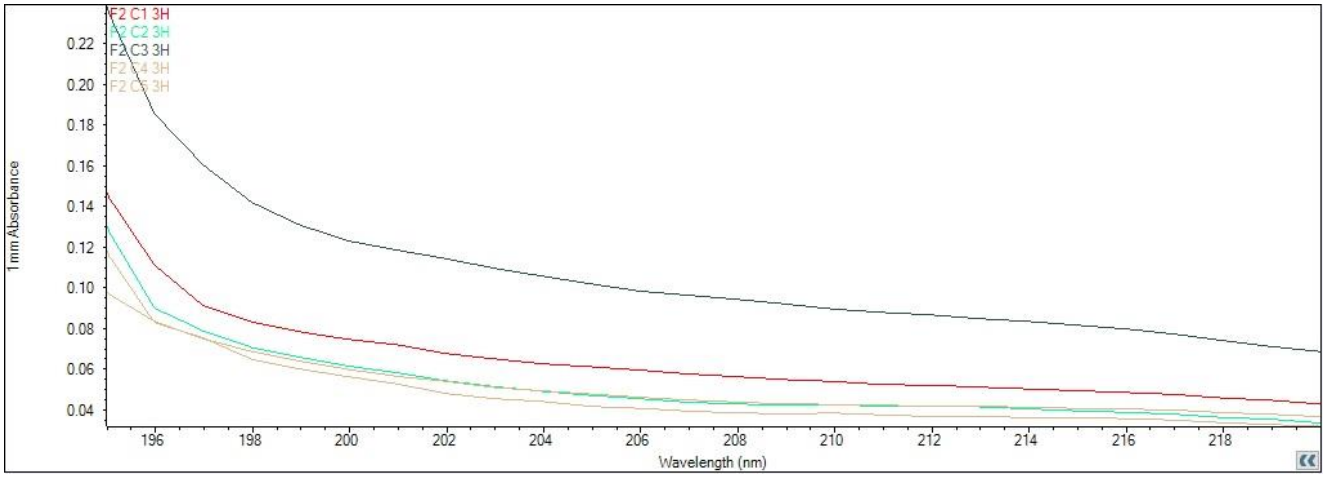
After 2h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.10542	0.10037	0.10949	0.10644	0.10356
$\mathbf{m}_{a,max}$	0.002120	0.002019	0.002202	0.002141	0.002083
$\mathbf{A}_{bl}$ (220 nm)	0.037	0.025	0.03	0.16	0.032
$\mathbf{A}_{max,a,t}$ (202 nm)	0.056	0.039	0.044	0.268	0.05
$\mathbf{c}_{a,t}$	29.38	26.21	26.21	85.78	28.75
$\mathbf{m}_{a,t}$	205.66	183.47	183.47	600.46	201.25
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	9.701	9.087	8.332	28.046	9.662

Spectra



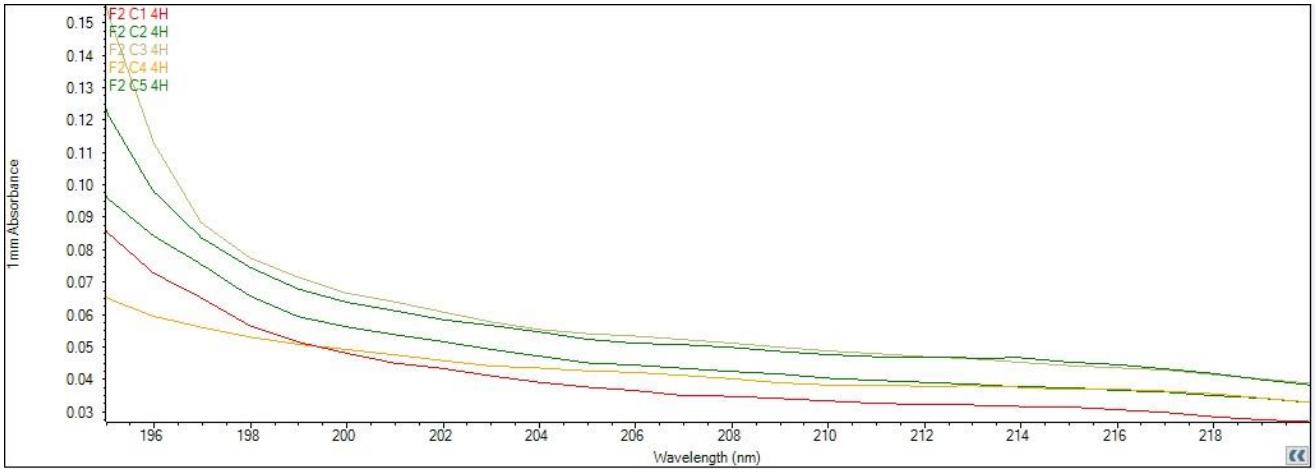
After 3h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10542	0.10037	0.10949	0.10644	0.10356
$m_{a,max}$	0.002120	0.002019	0.002202	0.002141	0.002083
A_{bl} (220 nm)	0.042	0.033	0.068	0.031	0.036
$A_{max,a,t}$ (202 nm)	0.067	0.054	0.114	0.047	0.053
$c_{a,t}$	33.18	30.65	46.49	27.48	28.11
$m_{a,t}$	232.26	214.55	325.43	192.36	196.77
$m_{a,t}/m_{a,max}$	10.956	10.627	14.779	8.985	9.447

Spectra



After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10542	0.10037	0.10949	0.10644	0.10356
$m_{a,max}$	0.002120	0.002019	0.002202	0.002141	0.002083
A_{bl} (220 nm)	0.027	0.033	0.038	0.033	0.038
$A_{max,a,t}$ (202 nm)	0.043	0.051	0.061	0.045	0.058
$c_{a,t}$	27.48	28.75	31.91	24.94	30.01
$m_{a,t}$	192.36	201.25	223.37	174.58	210.07
$m_{a,t}/m_{a,max}$	9.074	9.968	10.144	8.154	10.085

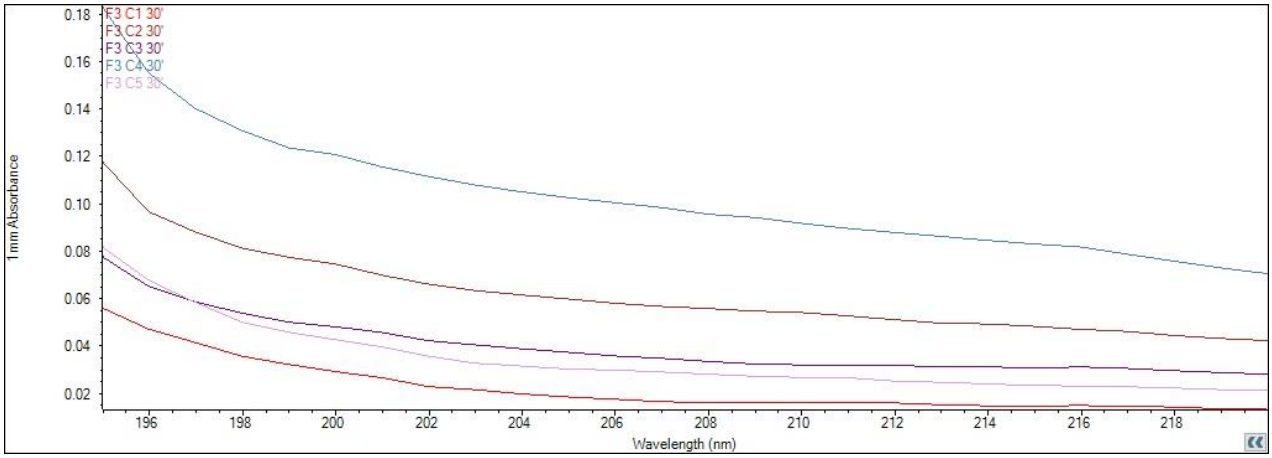
Spectra



Tuffryn® Membrane – GlcNAc + Glycerol + Water (2:1)

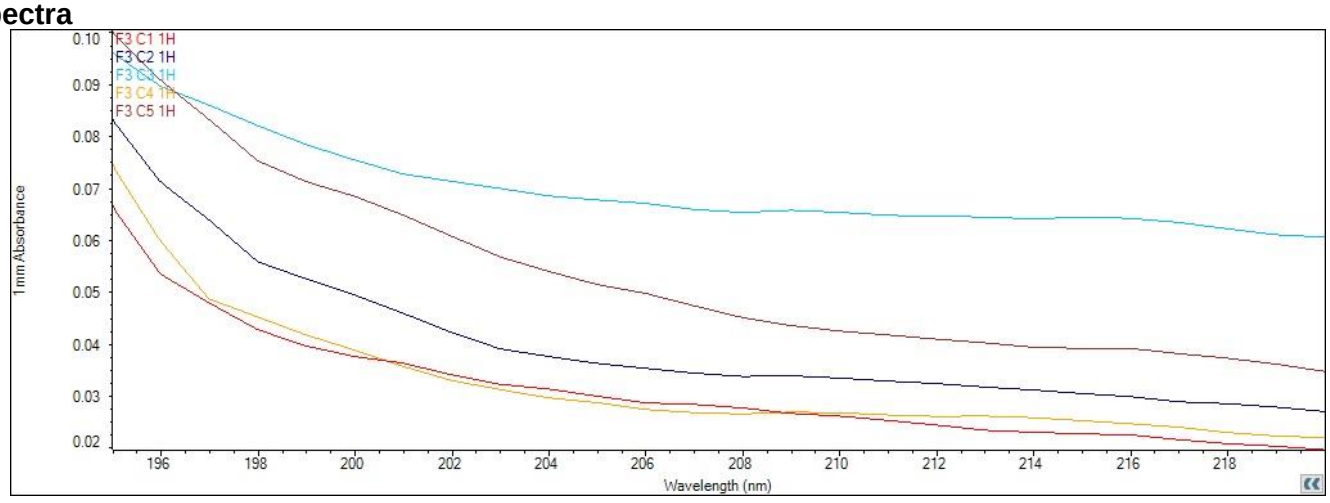
After 0.5h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.13189	0.11701	0.1071	0.09991	0.10477
$m_{a,max}$	0.002668	0.002367	0.002167	0.002021	0.002119
A_{bl} (220 nm)	0.013	0.042	0.028	0.07	0.021
$A_{max,a,t}$ (202 nm)	0.022	0.066	0.042	0.111	0.035
$c_{a,t}$	23.04	32.55	26.21	43.32	26.21
$m_{a,t}$	161.28	227.85	183.47	303.24	183.47
$m_{a,t}/m_{a,max}$	6.045	9.626	8.467	15.004	8.658

Spectra



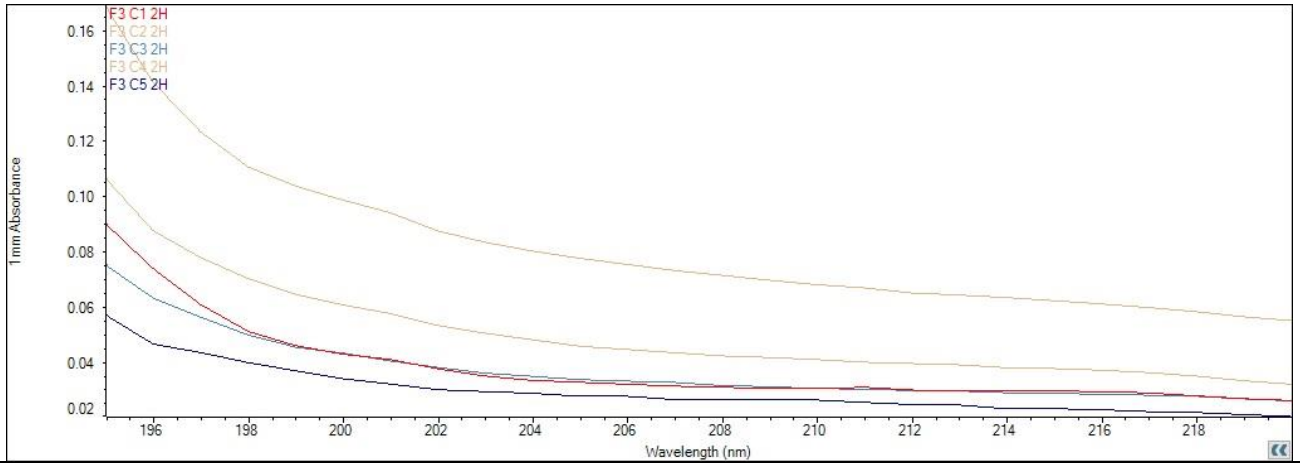
After 1h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.13189	0.11701	0.1071	0.09991	0.10477
$m_{a,max}$	0.002668	0.002367	0.002167	0.002021	0.002119
A_{bl} (220 nm)	0.019	0.027	0.061	0.022	0.035
$A_{max,a,t}$ (202 nm)	0.034	0.042	0.071	0.033	0.061
$c_{a,t}$	26.84	26.84	23.68	24.31	33.81
$m_{a,t}$	187.88	187.88	165.76	170.17	236.67
$m_{a,t}/m_{a,max}$	7.042	7.938	7.649	8.420	11.169

Spectra



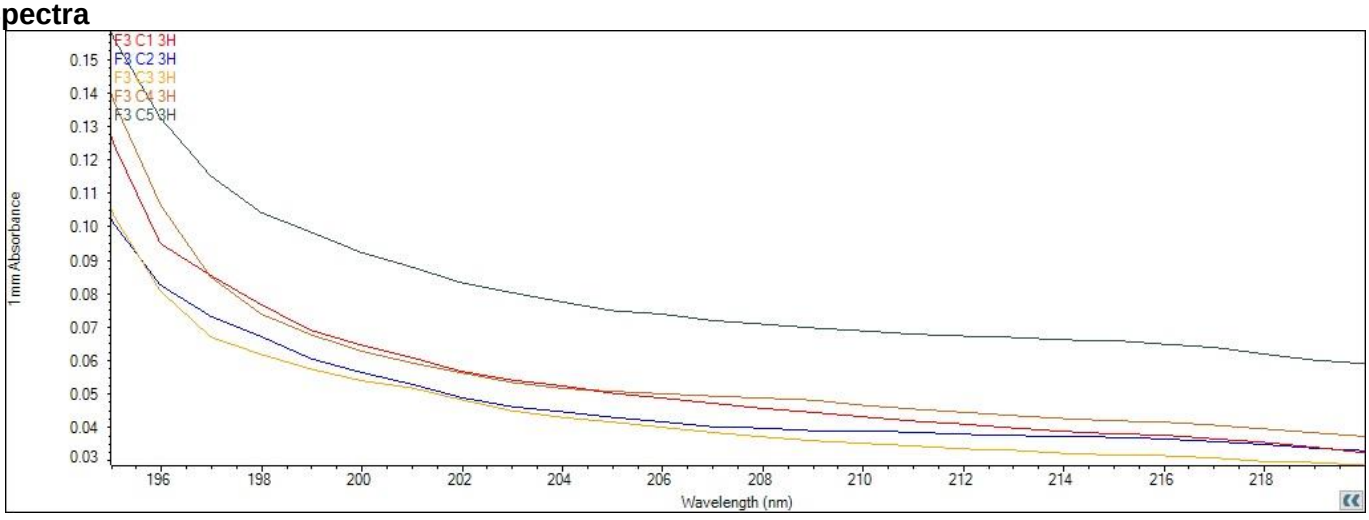
After 2h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.13189	0.11701	0.1071	0.09991	0.10477
$m_{a,max}$	0.002668	0.002367	0.002167	0.002021	0.002119
A_{bl} (220 nm)	0.026	0.055	0.026	0.032	0.02
$A_{max,a,t}$ (202 nm)	0.037	0.087	0.038	0.053	0.03
$c_{a,t}$	24.31	37.62	24.94	30.65	23.68
$m_{a,t}$	170.17	263.34	174.58	214.55	165.76
$m_{a,t}/m_{a,max}$	6.378	11.126	8.056	10.616	7.823

Spectra



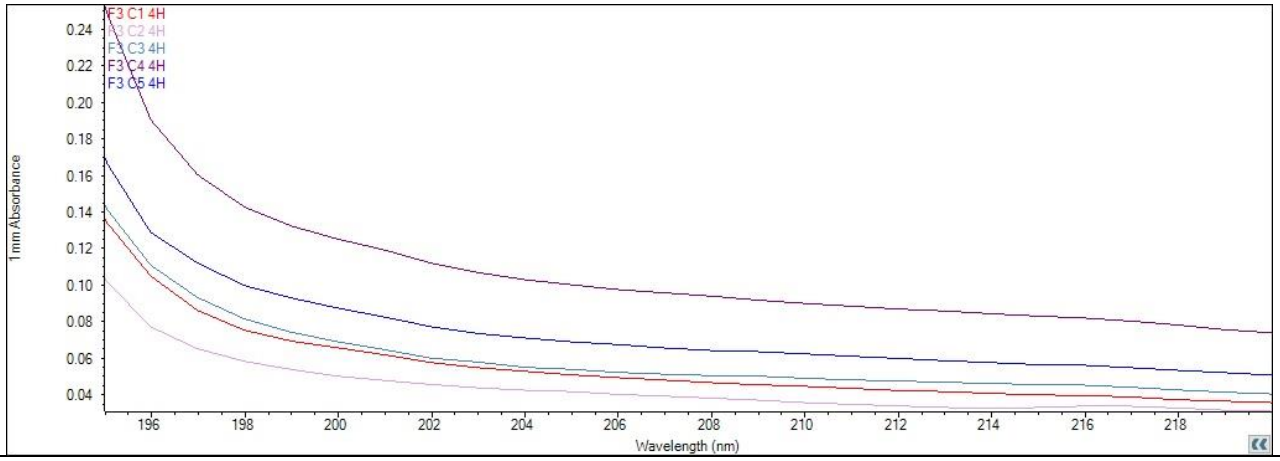
After 3h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.13189	0.11701	0.1071	0.09991	0.10477
$m_{a,max}$	0.002668	0.002367	0.002167	0.002021	0.002119
A_{bl} (220 nm)	0.032	0.033	0.028	0.037	0.059
$A_{max,a,t}$ (202 nm)	0.056	0.049	0.048	0.056	0.083
$c_{a,t}$	32.55	27.48	30.01	29.38	32.55
$m_{a,t}$	227.85	192.36	210.07	205.66	227.85
$m_{a,t}/m_{a,max}$	8.540	7.205	9.694	10.176	10.753

Spectra



After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.13189	0.11701	0.1071	0.09991	0.10477
$\mathbf{m}_{a,max}$	0.002668	0.002367	0.002167	0.002021	0.002119
$\mathbf{A}_{bl}$ (220 nm)	0.035	0.03	0.04	0.073	0.05
$\mathbf{A}_{max,a,t}$ (202 nm)	0.057	0.045	0.059	0.112	0.077
$\mathbf{c}_{a,t}$	31.28	26.84	29.38	42.05	34.45
$\mathbf{m}_{a,t}$	218.96	187.88	205.66	294.35	241.15
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	8.207	7.938	9.491	14.565	11.380

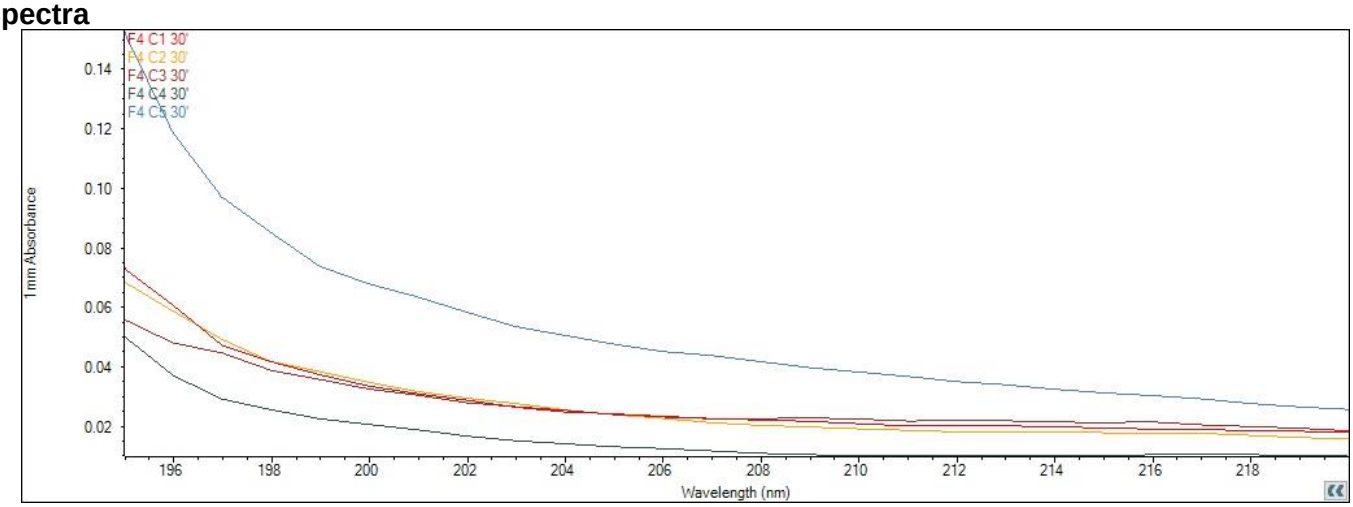
Spectra



Tuffryn® Membrane – GlcNAc + Glycerol + Water (1:2)

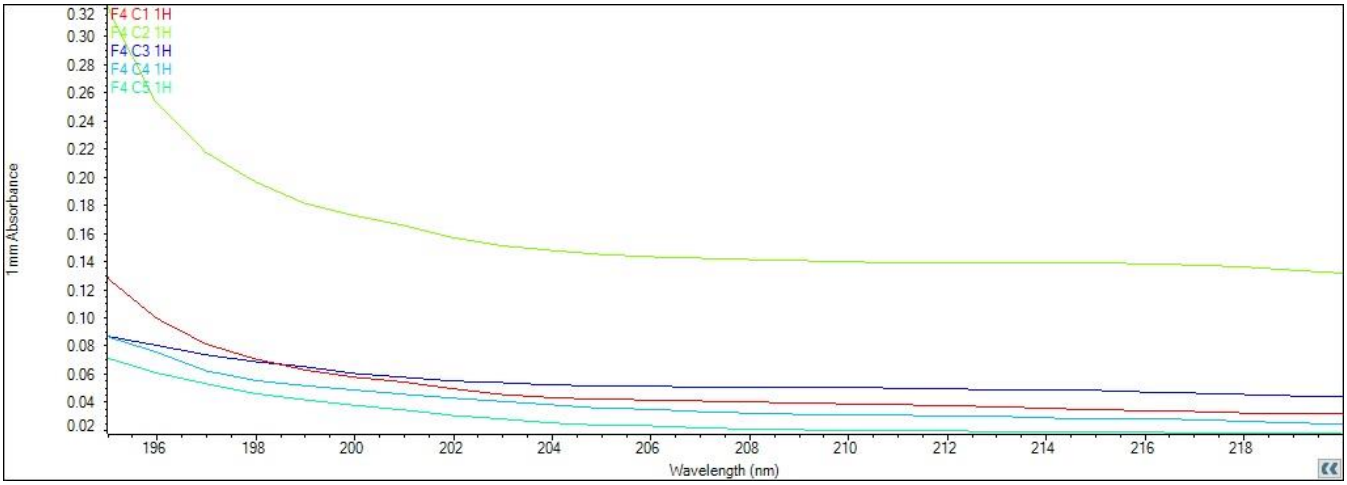
After 0.5h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11080	0.10282	0.11128	0.10024	0.10401
$\mathbf{m}_{a,max}$	0.02299	0.02133	0.02309	0.002080	0.02158
$\mathbf{A}_{bl}$ (220 nm)	0.018	0.015	0.018	0.01	0.025
$\mathbf{A}_{max,a,t}$ (202 nm)	0.028	0.029	0.028	0.016	0.058
$\mathbf{c}_{a,t}$	23.68	26.21	23.68	21.14	38.25
$\mathbf{m}_{a,t}$	165.76	183.47	165.76	147.98	267.75
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	7.210	8.602	7.179	7.114	12.407

Spectra



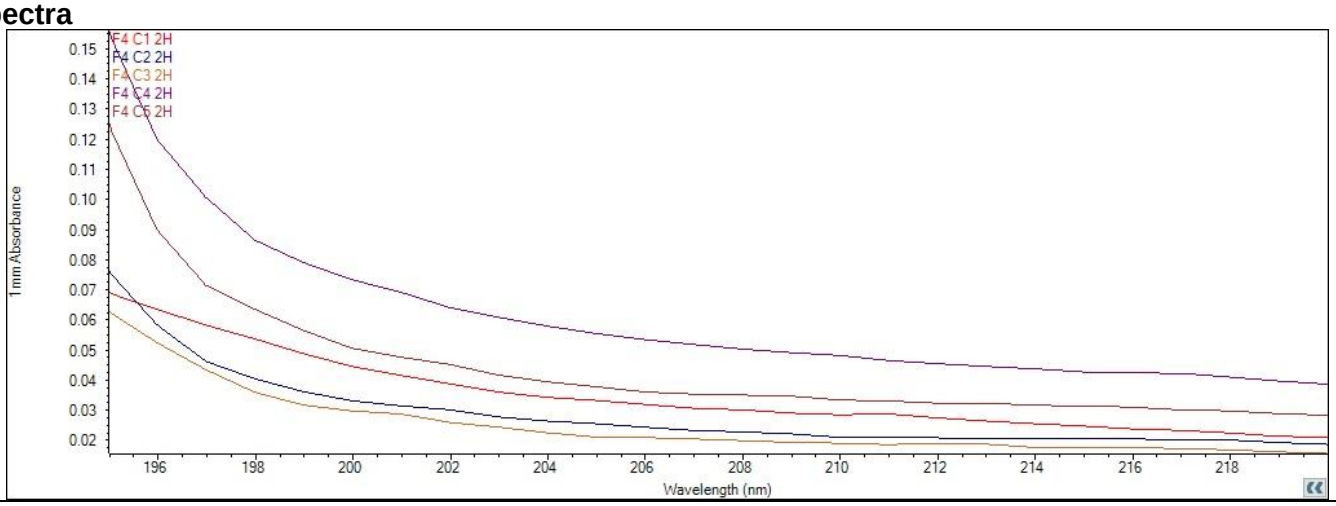
After 1h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11080	0.10282	0.11128	0.10024	0.10401
$\mathbf{m}_{a,max}$	0.02299	0.02133	0.02309	0.002080	0.02158
$\mathbf{A}_{bl}$ (220 nm)	0.031	0.131	0.043	0.024	0.017
$\mathbf{A}_{max,a,t}$ (202 nm)	0.049	0.156	0.054	0.042	0.03
$\mathbf{c}_{a,t}$	28.75	33.18	24.31	28.75	25.58
$\mathbf{m}_{a,t}$	201.25	232.26	170.17	201.25	179.06
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	8.754	10.889	7.370	9.671	8.298

Spectra



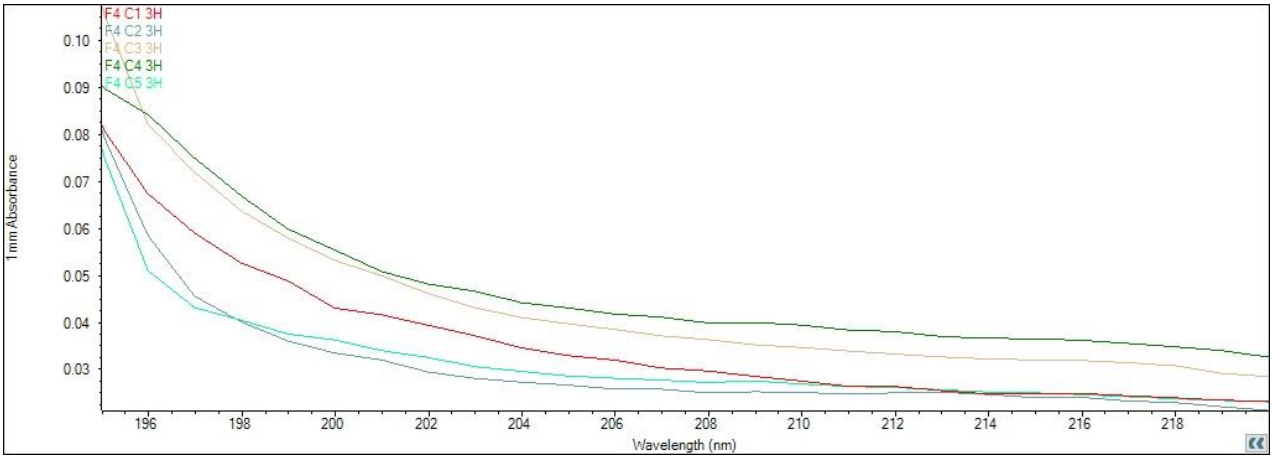
After 2h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11080	0.10282	0.11128	0.10024	0.10401
$\mathbf{m}_{a,max}$	0.02299	0.02133	0.02309	0.002080	0.02158
$\mathbf{A}_{bl}$ (220 nm)	0.021	0.018	0.015	0.038	0.028
$\mathbf{A}_{max,a,t}$ (202 nm)	0.038	0.03	0.026	0.064	0.045
$\mathbf{c}_{a,t}$	28.11	24.94	24.31	33.81	28.11
$\mathbf{m}_{a,t}$	196.77	174.58	170.17	236.67	196.77
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	8.559	8.185	7.370	11.378	9.118

Spectra



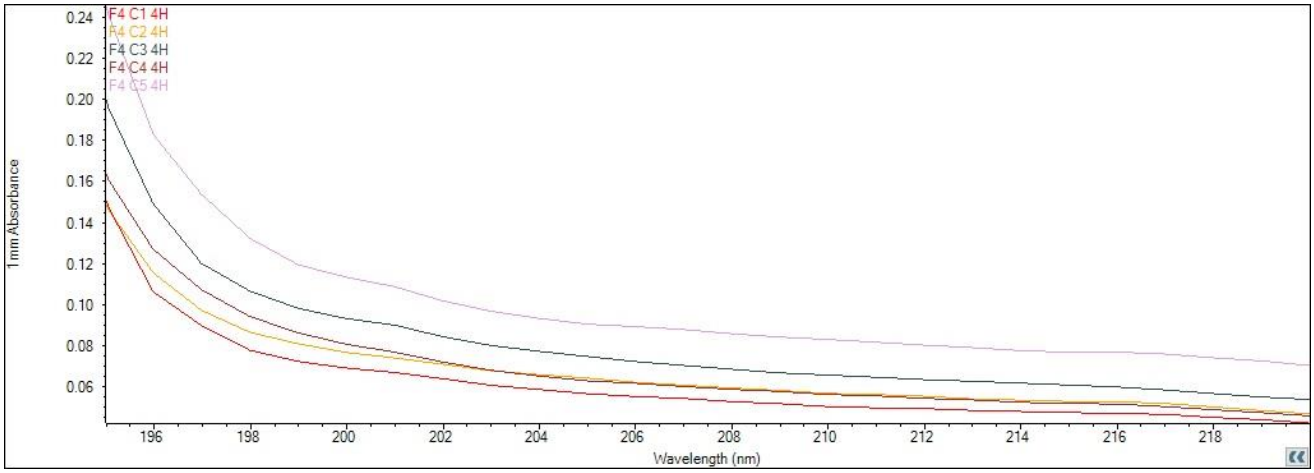
After 3h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11080	0.10282	0.11128	0.10024	0.10401
$\mathbf{m}_{a,max}$	0.02299	0.02133	0.02309	0.002080	0.02158
$\mathbf{A}_{bl}$ (220 nm)	0.023	0.021	0.028	0.032	0.023
$\mathbf{A}_{max,a,t}$ (202 nm)	0.039	0.029	0.046	0.048	0.032
$\mathbf{c}_{a,t}$	27.48	22.41	28.75	27.48	23.04
$\mathbf{m}_{a,t}$	192.36	156.87	201.25	192.36	161.28
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	8.367	7.354	8.716	9.248	7.474

Spectra



After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.11080	0.10282	0.11128	0.10024	0.10401
$\mathbf{m}_{a,max}$	0.02299	0.02133	0.02309	0.002080	0.02158
$\mathbf{A}_{bl}$ (220 nm)	0.042	0.046	0.053	0.045	0.07
$\mathbf{A}_{max,a,t}$ (202 nm)	0.064	0.071	0.084	0.072	0.102
$\mathbf{c}_{a,t}$	31.28	33.18	36.98	34.45	37.62
$\mathbf{m}_{a,t}$	218.96	232.26	258.86	241.15	263.34
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	9.524	10.889	11.211	11.594	12.203

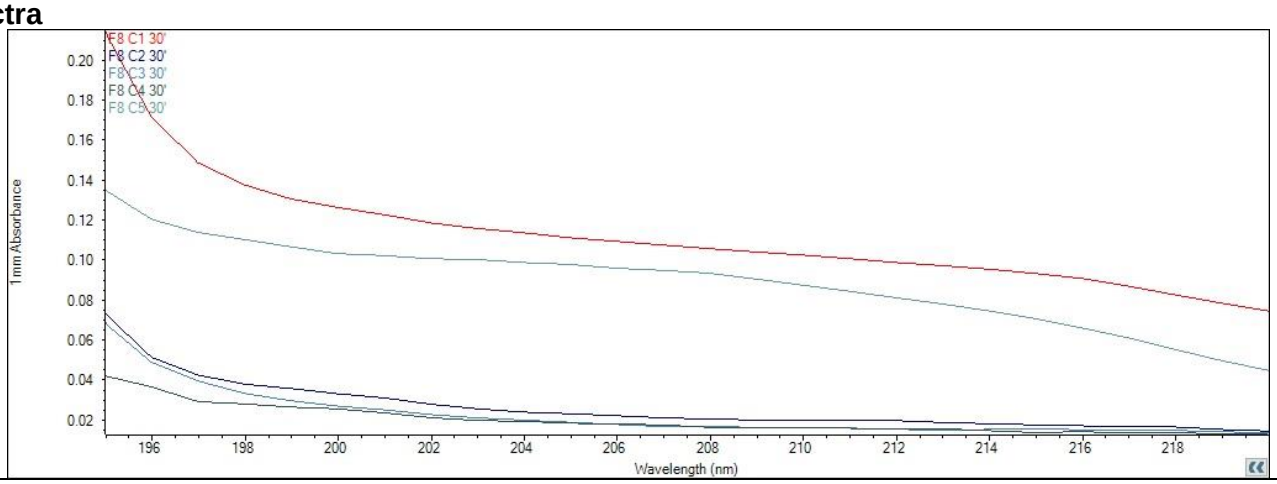
Spectra



Tuffryn® Membrane – GlcNAc + Glycerol + Propylen glycol + Water (1:1:1)

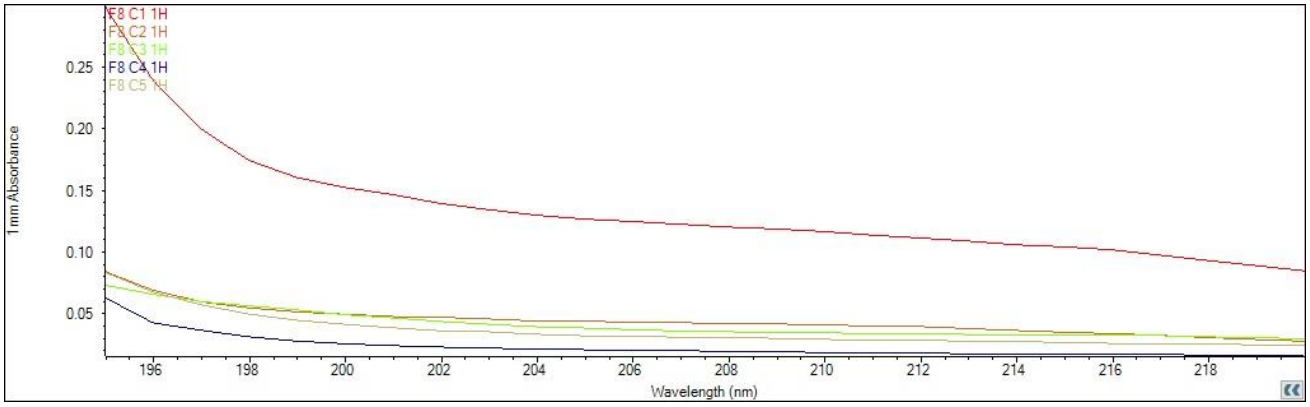
After 0.5h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10880	0.10675	0.10541	0.11908	0.11944
$m_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
A_{bl} (220 nm)	0.074	0.014	0.013	0.012	0.044
$A_{max,a,t}$ (202 nm)	0.118	0.027	0.022	0.021	0.1
$c_{a,t}$	45.22	25.58	23.04	23.04	52.83
$m_{a,t}$	316.54	179.06	161.28	161.28	369.81
$m_{a,t}/m_{a,max}$	14.547	8.387	7.647	6.771	15.480

Spectra



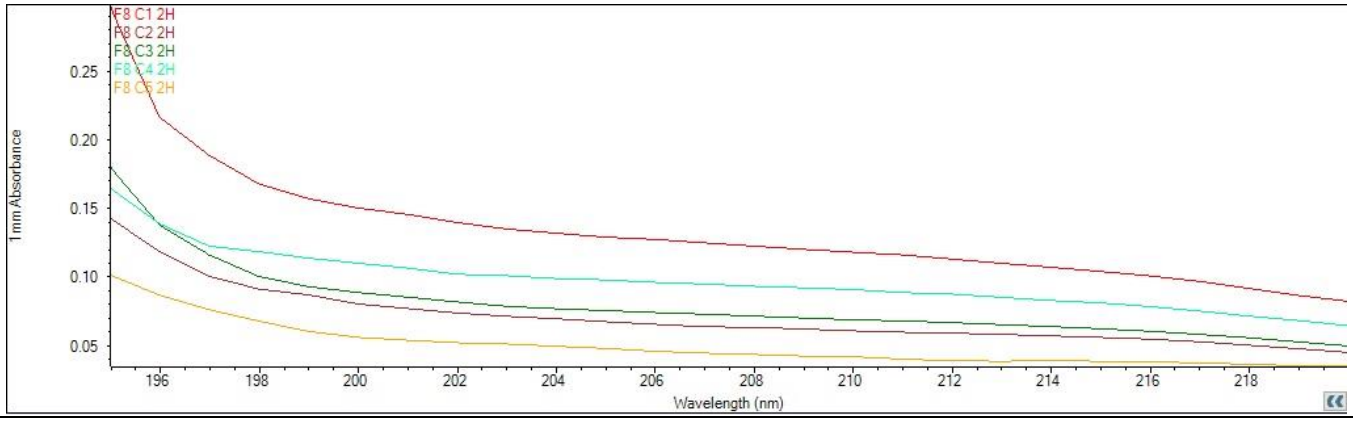
After 1h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10880	0.10675	0.10541	0.11908	0.11944
$m_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
A_{bl} (220 nm)	0.084	0.027	0.029	0.015	0.023
$A_{max,a,t}$ (202 nm)	0.139	0.046	0.043	0.022	0.035
$c_{a,t}$	52.19	29.38	26.21	21.77	24.94
$m_{a,t}$	365.33	205.66	183.47	152.39	174.58
$m_{a,t}/m_{a,max}$	16.789	9.633	8.699	6.398	7.308

Spectra



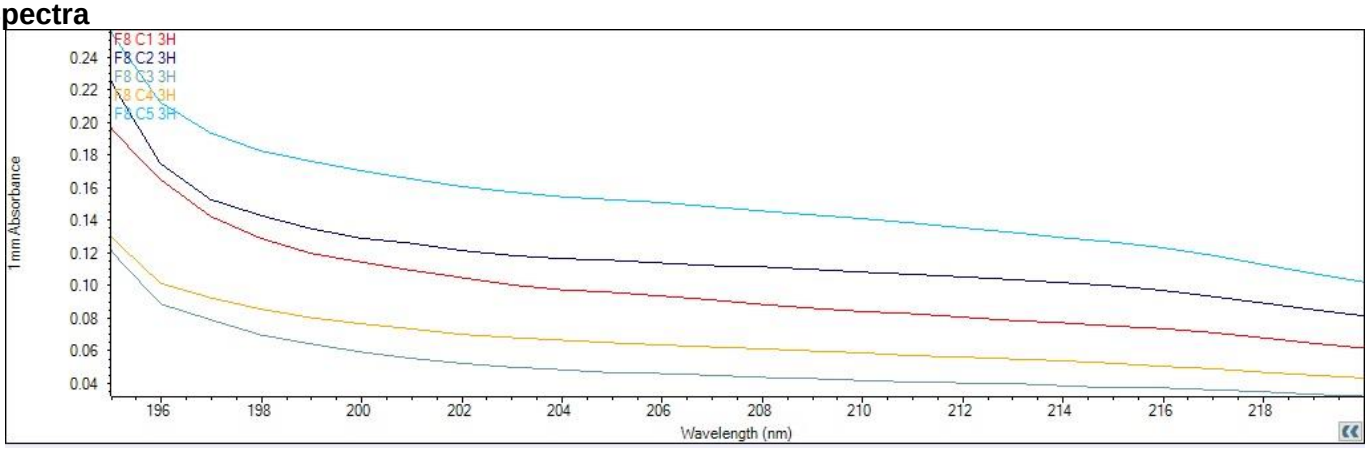
After 2h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10880	0.10675	0.10541	0.11908	0.11944
$m_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
A_{bl} (220 nm)	0.082	0.044	0.049	0.064	0.034
$A_{max,a,t}$ (202 nm)	0.139	0.073	0.081	0.101	0.051
$c_{a,t}$	53.46	35.72	37.62	40.79	28.11
$m_{a,t}$	374.22	250.04	263.34	285.53	196.77
$m_{a,t}/m_{a,max}$	17.198	11.711	12.486	11.987	8.237

Spectra



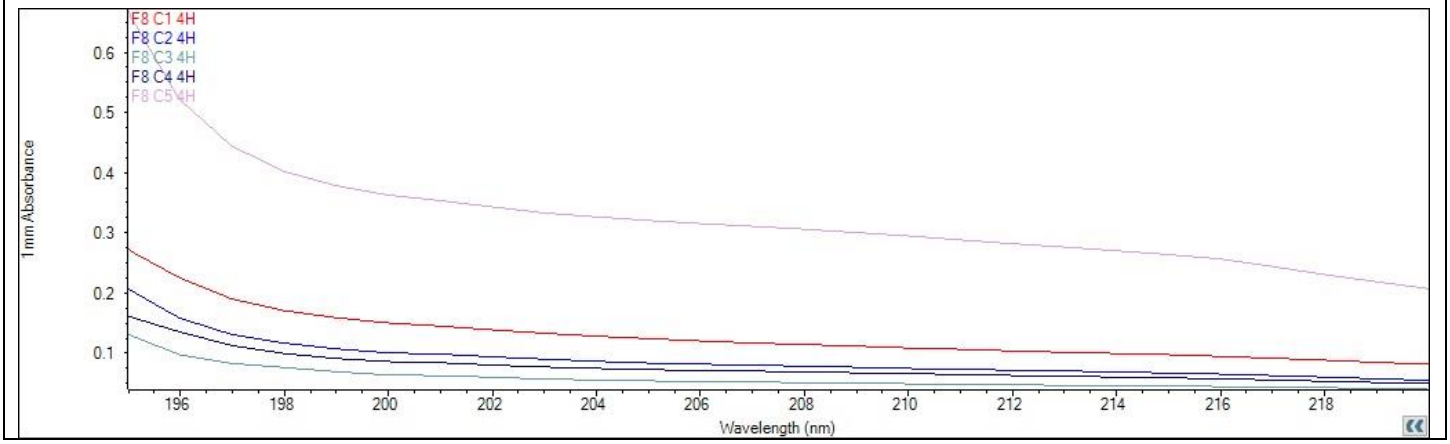
After 3h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10880	0.10675	0.10541	0.11908	0.11944
$m_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
A_{bl} (220 nm)	0.061	0.081	0.031	0.043	0.102
$A_{max,a,t}$ (202 nm)	0.104	0.121	0.052	0.069	0.16
$c_{a,t}$	44.59	42.69	30.65	33.81	54.09
$m_{a,t}$	312.13	298.83	214.55	236.67	378.63
$m_{a,t}/m_{a,max}$	14.344	13.997	10.173	9.936	15.849

Spectra



After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
$\mathbf{m}_{form}$	0.10880	0.10675	0.10541	0.11908	0.11944
$\mathbf{m}_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
$\mathbf{A}_{bl}$ (220 nm)	0.08	0.053	0.038	0.047	0.206
$\mathbf{A}_{max,a,t}$ (202 nm)	0.137	0.092	0.058	0.078	0.342
$\mathbf{c}_{a,t}$	53.46	42.05	30.01	36.98	103.52
$\mathbf{m}_{a,t}$	372.22	294.35	210.07	258.86	724.64
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	17.106	13.787	9.961	10.867	30.332

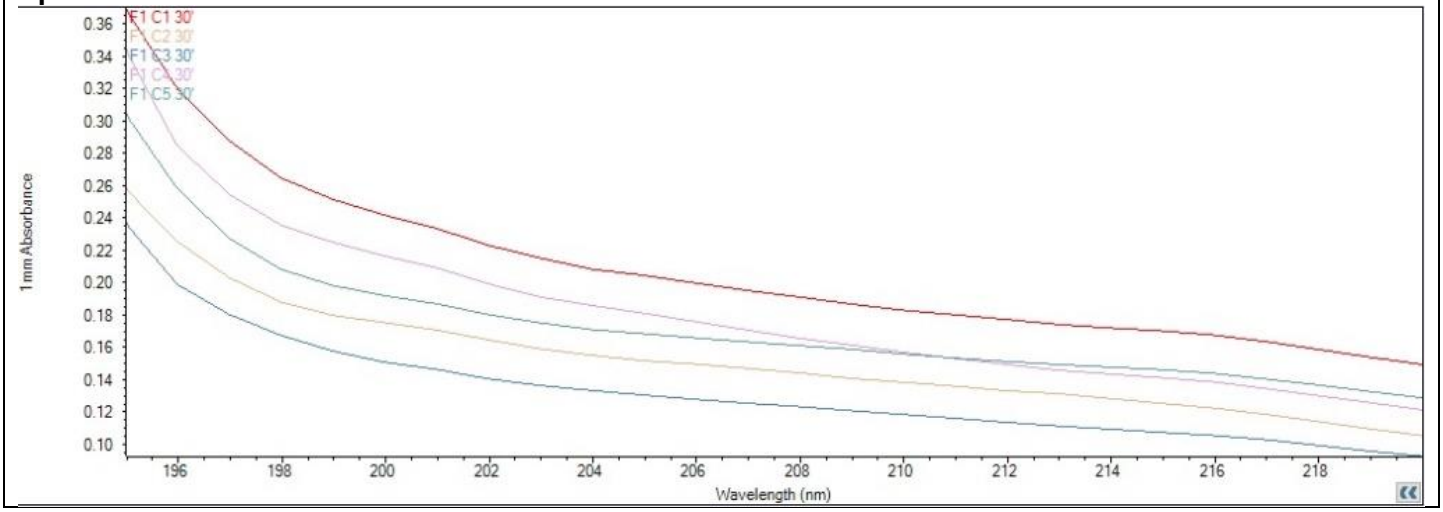
Spectra



Strat-M® Membrane – GlcNAc + Water

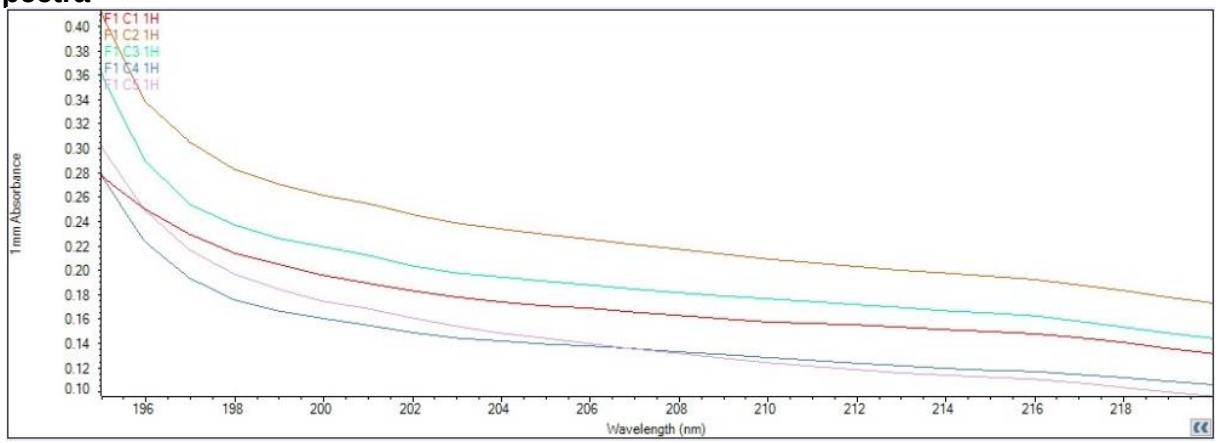
After 0,5h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10415	0.10627	0.10432
$\mathbf{m}_{a,max}$	0.002169	0.002213	0.002173
$\mathbf{A}_{bl}$ (220 nm)	0.040	0.016	0.016
$\mathbf{A}_{max,a,t}$ (202 nm)	0.083	0.004	0.034
$\mathbf{c}_{a,t}$	44.59	32.55	28.75
$\mathbf{m}_{a,t}$	312.13	227.85	201.25
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	14.391	10.296	9.261

Spectra



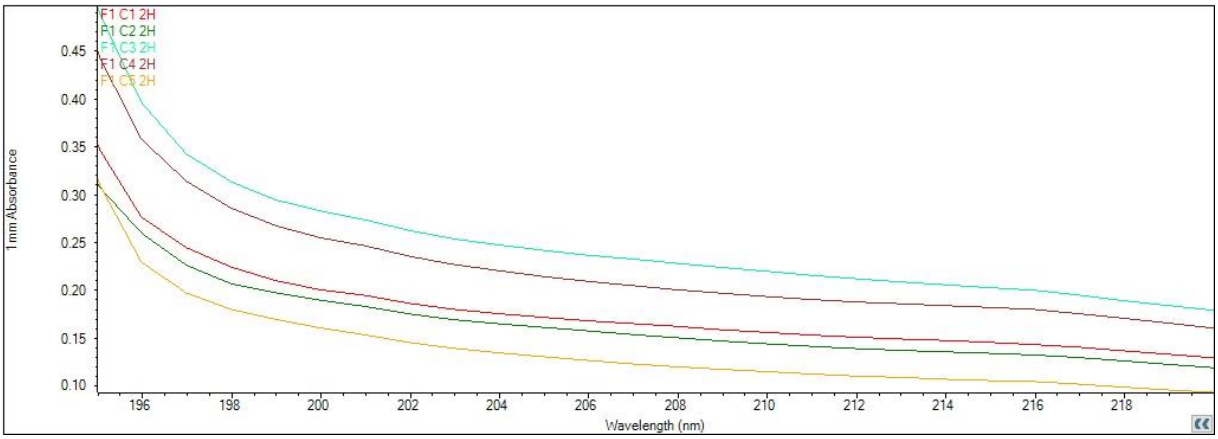
After 1h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10415	0.10627	0.10432
$m_{a,max}$	0.002169	0.002213	0.002173
A_{bl} (220 nm)	0.017	0.010	0.010
$A_{max,a,t}$ (202 nm)	0.040	0.025	0.023
$c_{a,t}$	31.91	26.84	25.58
$m_{a,t}$	223.37	187.88	179.06
$m_{a,t}/m_{a,max}$	10.298	8.490	8.240

Spectra



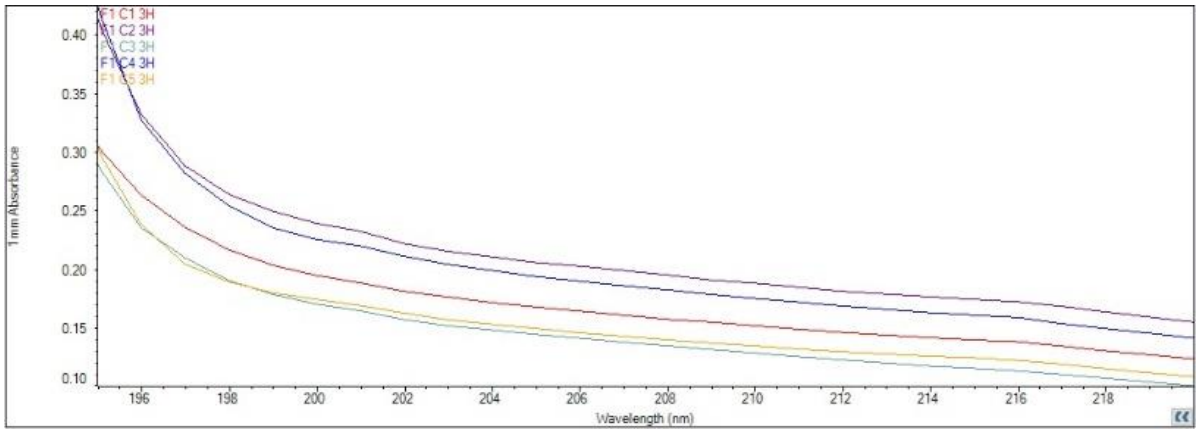
After 2h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10415	0.10627	0.10432
$m_{a,max}$	0.002169	0.002213	0.002173
A_{bl} (220 nm)	0.010	0.014	0.005
$A_{max,a,t}$ (202 nm)	0.023	0.024	0.011
$c_{a,t}$	25.58	23.68	21.14
$m_{a,t}$	179.06	165.76	147.98
$m_{a,t}/m_{a,max}$	8.255	7.490	6.810

Spectra



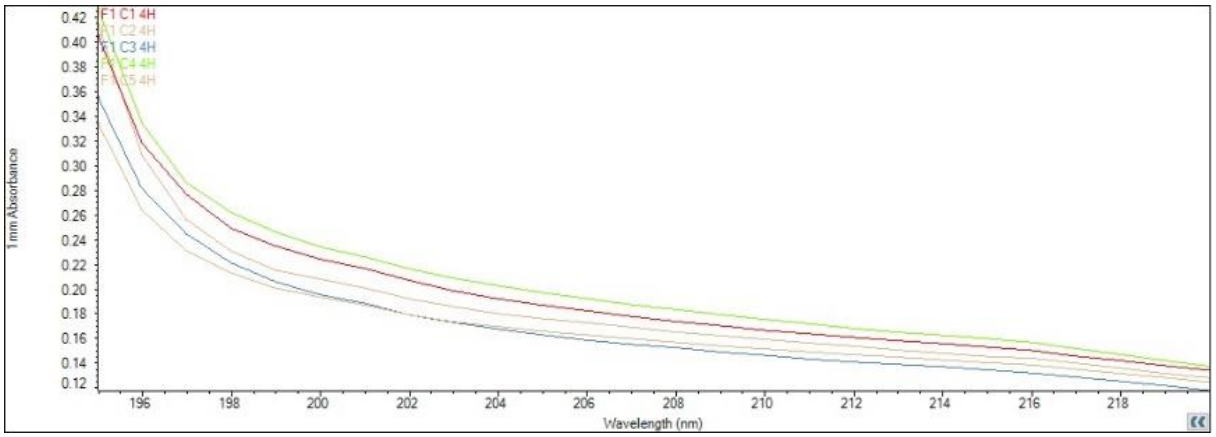
After 3h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10415	0.10627	0.10432
$m_{a,max}$	0.002169	0.002213	0.002173
A_{bl} (220 nm)	0.010	0.005	0.007
$A_{max,a,t}$ (202 nm)	0.020	0.012	0.014
$c_{a,t}$	23.68	21.77	21.77
$m_{a,t}$	165.76	152.39	152.39
$m_{a,t}/m_{a,max}$	7.642	6.886	7.013

Spectra



After 4h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10415	0.10627	0.10432
$m_{a,max}$	0.002169	0.002213	0.002173
A_{bl} (220 nm)	0.003	0.010	0.002
$A_{max,a,t}$ (202 nm)	0.010	0.016	0.009
$c_{a,t}$	21.77	21.14	21.77
$m_{a,t}$	152.39	147.98	152.39
$m_{a,t}/m_{a,max}$	7.026	6.687	7.013

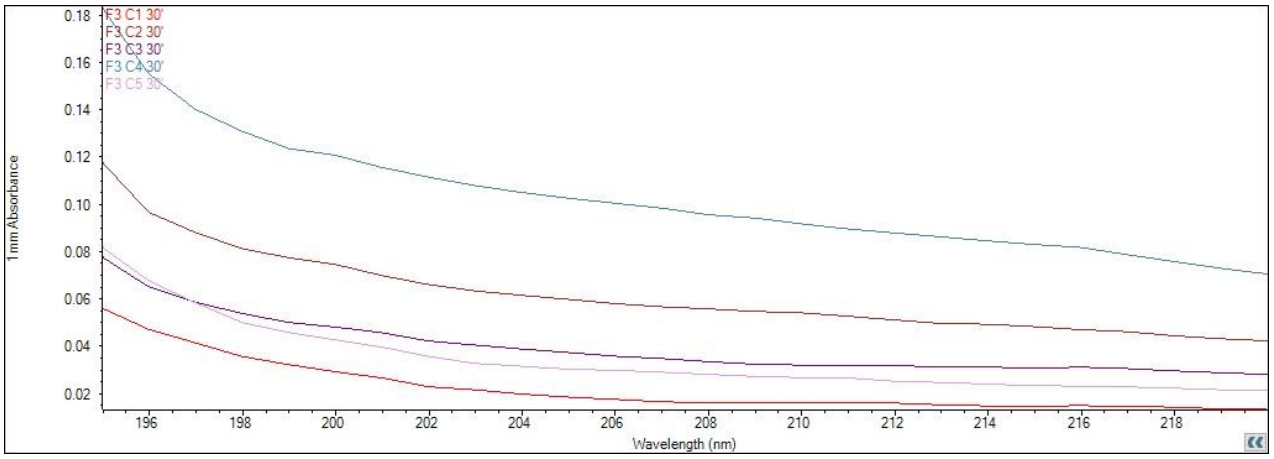
Spectra



Strat-M® Membrane – GlcNAc + Glycerol + Water (2:1)

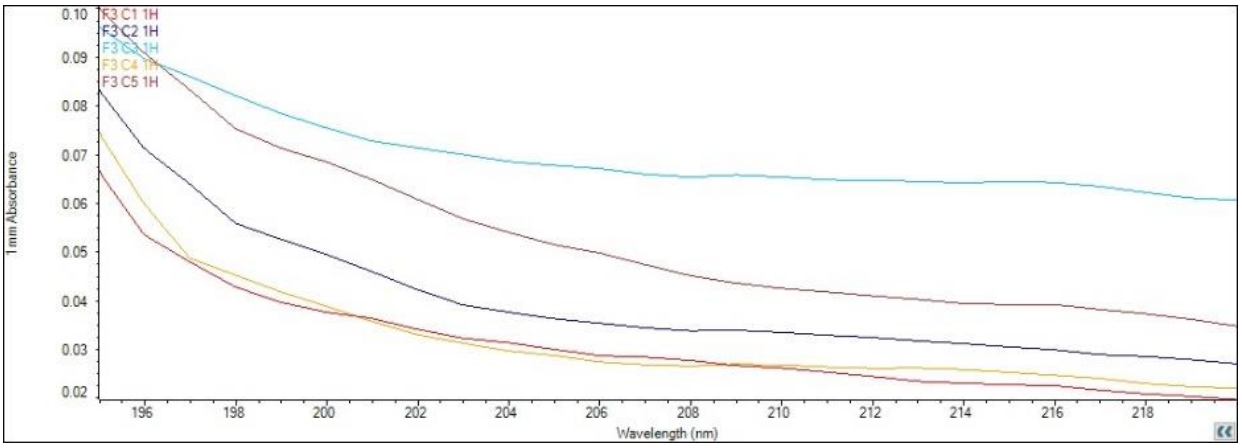
After 0.5h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10330	0.11234	0.10331
$m_{a,max}$	0.002090	0.002273	0.002090
A_{bl} (220 nm)	0.007	0.015	0.015
$A_{max,a,t}$ (202 nm)	0.026	0.038	0.030
$c_{a,t}$	29.38	31.91	26.84
$m_{a,t}$	205.66	223.37	187.88
$m_{a,t}/m_{a,max}$	9.840	9.827	8.989

Spectra



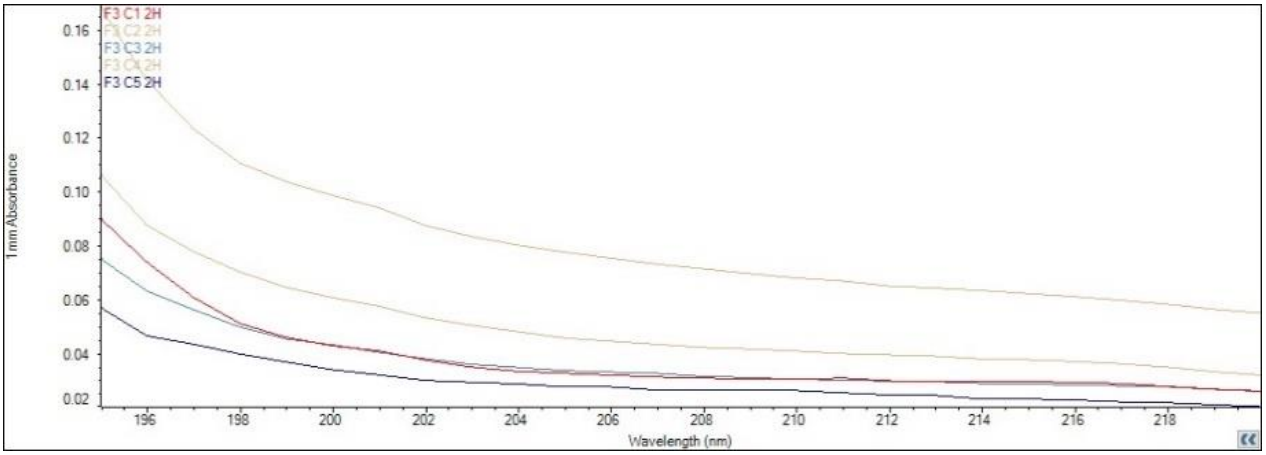
After 1h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10330	0.11234	0.10331
$m_{a,max}$	0.002090	0.002273	0.002090
A_{bl} (220 nm)	0.003	0.005	0.007
$A_{max,a,t}$ (202 nm)	0.027	0.019	0.019
$c_{a,t}$	32.55	26.21	24.94
$m_{a,t}$	227.85	183.47	174.58
$m_{a,t}/m_{a,max}$	10.902	8.072	8.353

Spectra



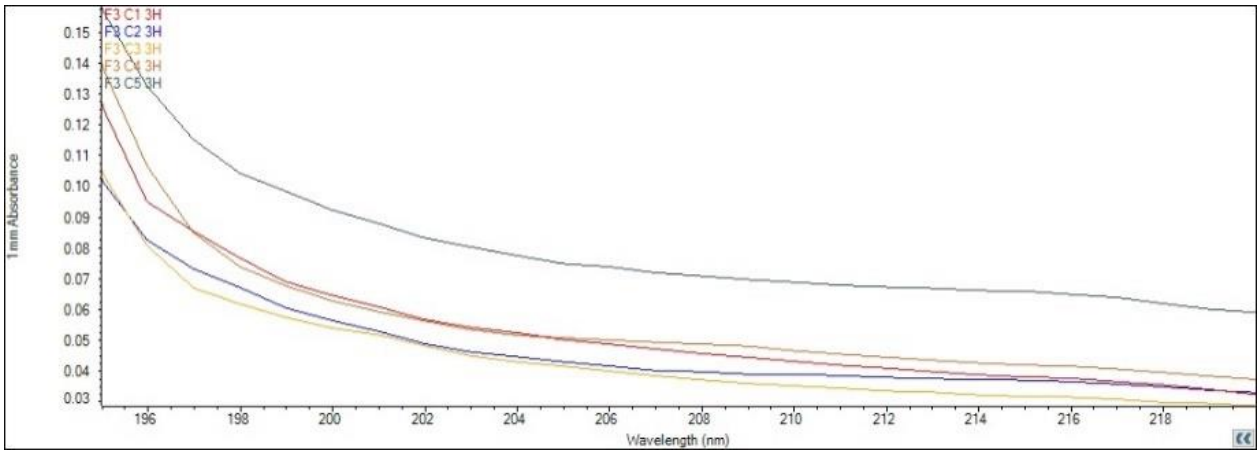
After 2h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10330	0.11234	0.10331
$m_{a,max}$	0.002090	0.002273	0.002090
A_{bl} (220 nm)	0.007	0.019	0.019
$A_{max,a,t}$ (202 nm)	0.014	0.049	0.051
$c_{a,t}$	21.77	36.45	37.62
$m_{a,t}$	152.39	255.15	263.34
$m_{a,t}/m_{a,max}$	7.291	11.225	12.600

Spectra



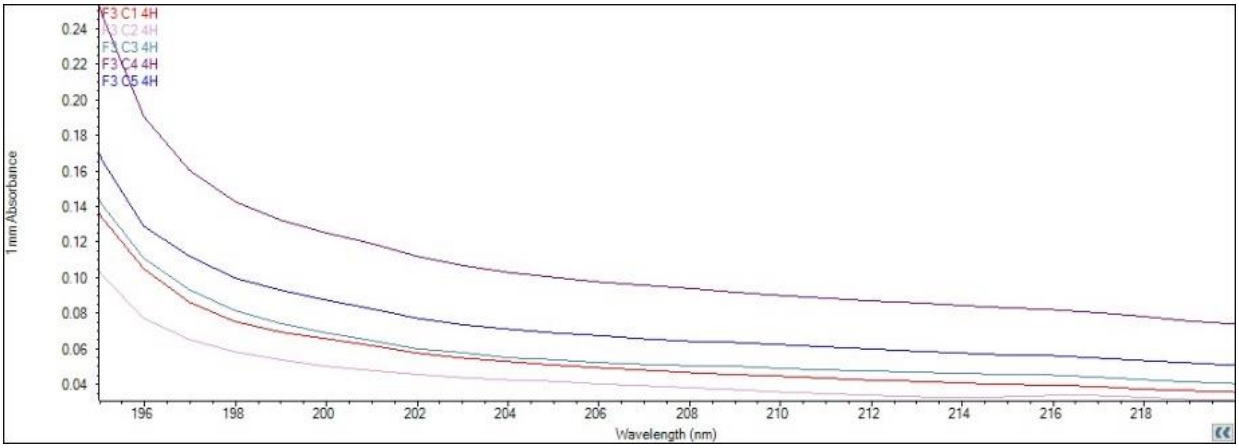
After 3h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10330	0.11234	0.10331
$m_{a,max}$	0.002090	0.002273	0.002090
A_{bl} (220 nm)	0.001	0.008	0.003
$A_{max,a,t}$ (202 nm)	0.005	0.021	0.012
$c_{a,t}$	19.87	25.58	23.04
$m_{a,t}$	139.09	179.06	161.28
$m_{a,t}/m_{a,max}$	6.655	7.878	7.717

Spectra



After 4h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10330	0.11234	0.10331
$\mathbf{m}_{a,max}$	0.002090	0.002273	0.002090
$\mathbf{A}_{bl}$ (220 nm)	0.011	0.007	0.009
$\mathbf{A}_{max,a,t}$ (202 nm)	0.021	0.015	0.022
$\mathbf{c}_{a,t}$	23.68	22.41	29.38
$\mathbf{m}_{a,t}$	165.76	187.91	205.65
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	7.931	8.267	9.840

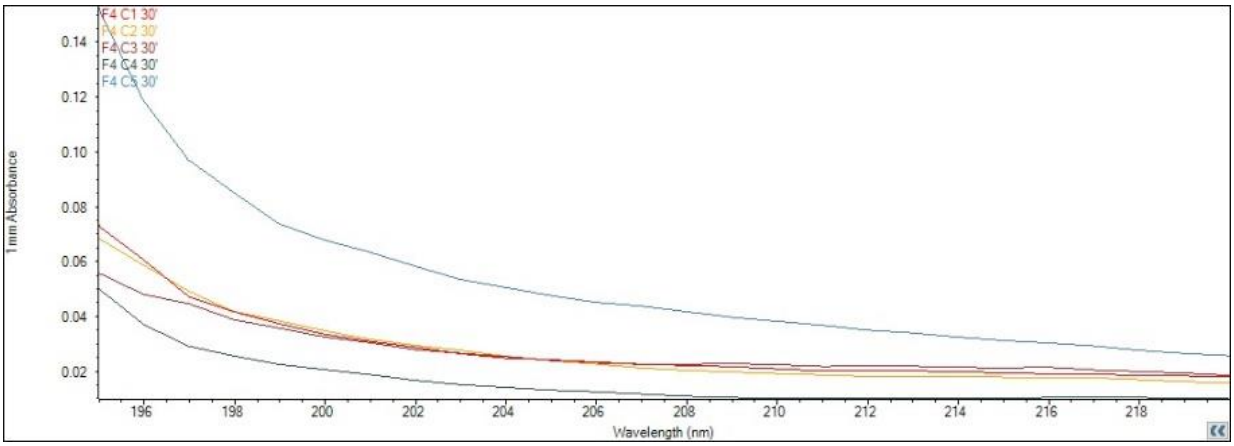
Spectra



Strat-M® Membrane – GlcNAc + Glycerol + Water (1:2)

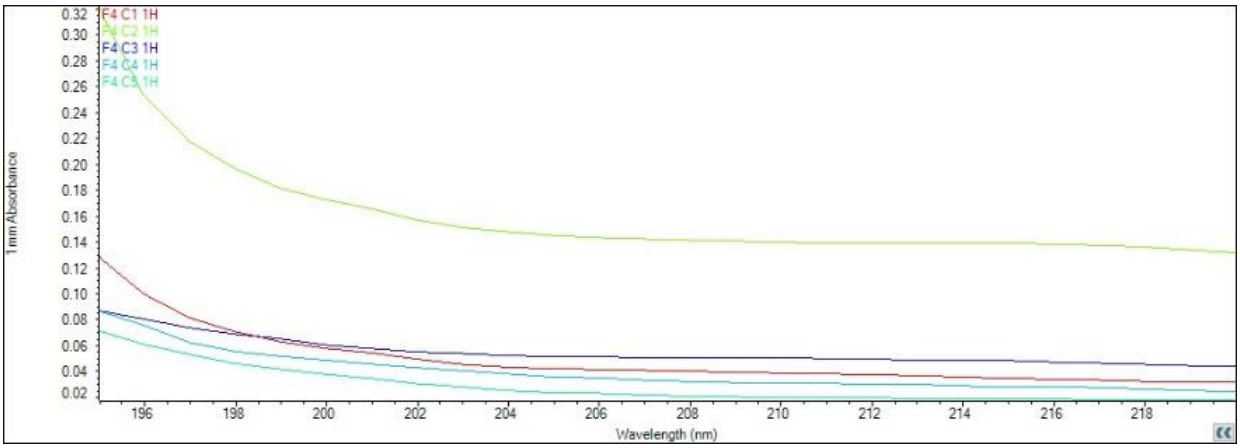
After 0.5h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10520	0.10462	0.10080
$\mathbf{m}_{a,max}$	0.02183	0.02171	0.02091
$\mathbf{A}_{bl}$ (220 nm)	0.009	0.025	0.018
$\mathbf{A}_{max,a,t}$ (202 nm)	0.028	0.056	0.046
$\mathbf{c}_{a,t}$	29.38	36.98	35.08
$\mathbf{m}_{a,t}$	205.66	258.86	245.56
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	9.421	11.924	11.744

Spectra



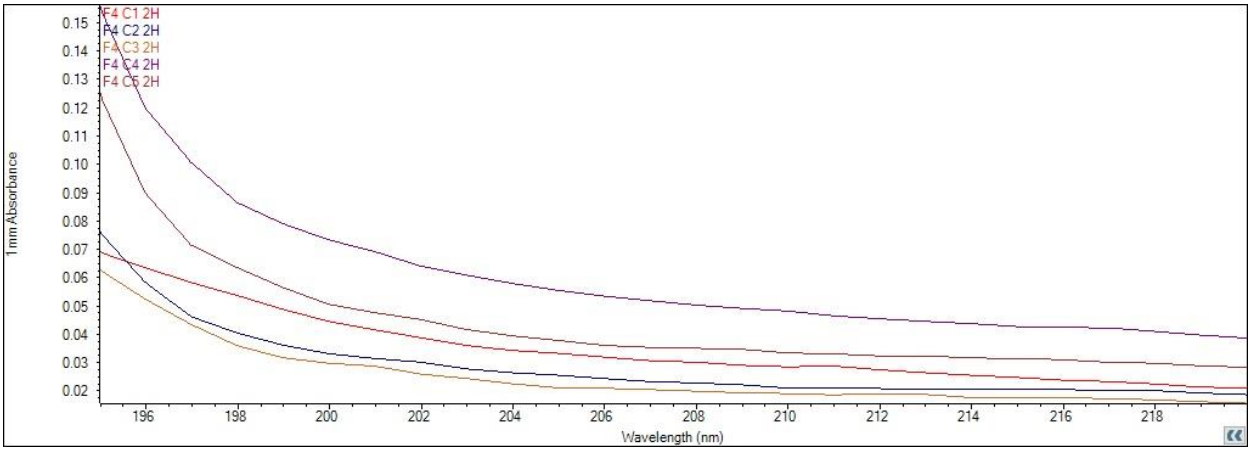
After 1h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10520	0.10462	0.10080
$m_{a,max}$	0.02183	0.02171	0.02091
A_{bl} (220 nm)	0.001	0.004	-0.003
$A_{max,a,t}$ (202 nm)	0.007	0.012	0.006
$c_{a,t}$	21.14	22.41	23.04
$m_{a,t}$	147.98	156.87	161.28
$m_{a,t}/m_{a,max}$	6.779	7.226	7.713

Spectra



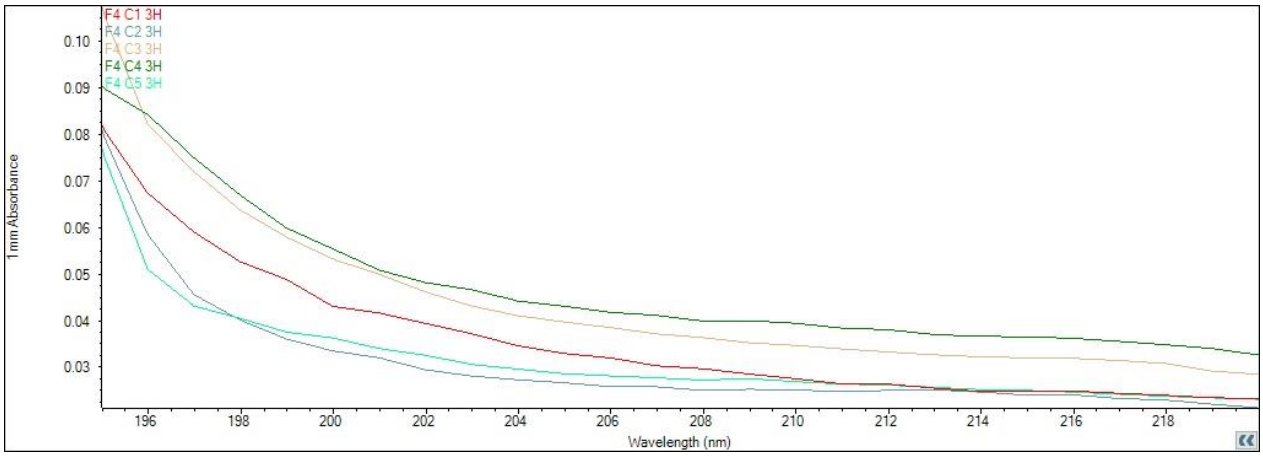
After 2h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10520	0.10462	0.10080
$m_{a,max}$	0.02183	0.02171	0.02091
A_{bl} (220 nm)	0.008	0.004	-0.001
$A_{max,a,t}$ (202 nm)	0.014	0.011	0.007
$c_{a,t}$	21.14	21.77	22.41
$m_{a,t}$	147.98	152.39	156.87
$m_{a,t}/m_{a,max}$	6.779	7.019	7.502

Spectra



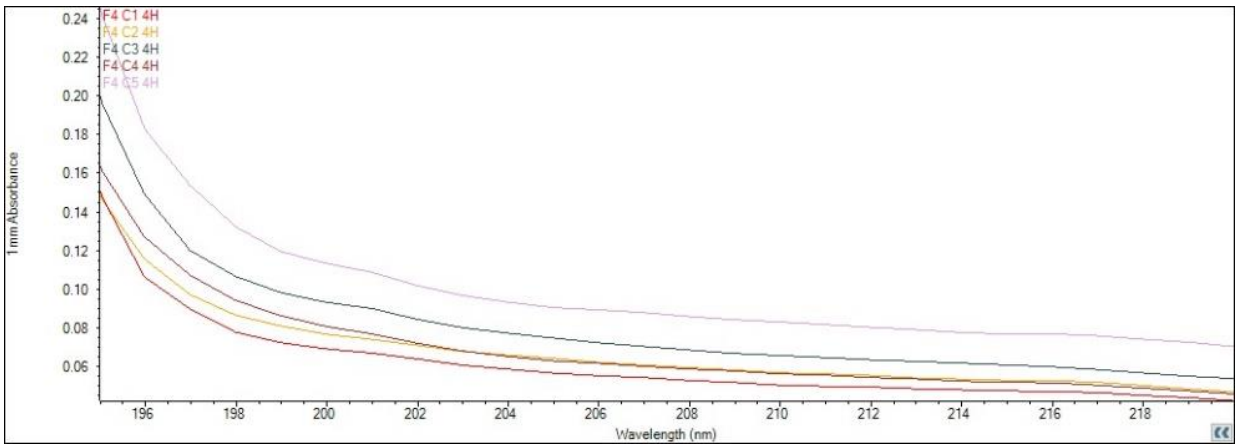
After 3h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10520	0.10462	0.10080
$m_{a,max}$	0.02183	0.02171	0.02091
A_{bl} (220 nm)	0.004	0.008	0.003
$A_{max,a,t}$ (202 nm)	0.013	0.016	0.015
$c_{a,t}$	23.04	22.41	24.94
$m_{a,t}$	161.28	156.86	174.58
$m_{a,t}/m_{a,max}$	7.388	7.225	8.349

Spectra



After 4h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10520	0.10462	0.10080
$m_{a,max}$	0.02183	0.02171	0.02091
A_{bl} (220 nm)	0.012	0.002	0.015
$A_{max,a,t}$ (202 nm)	0.021	0.002	0.017
$c_{a,t}$	23.04	17.34	18.61
$m_{a,t}$	161.28	121.38	130.27
$m_{a,t}/m_{a,max}$	7.388	5.591	6.230

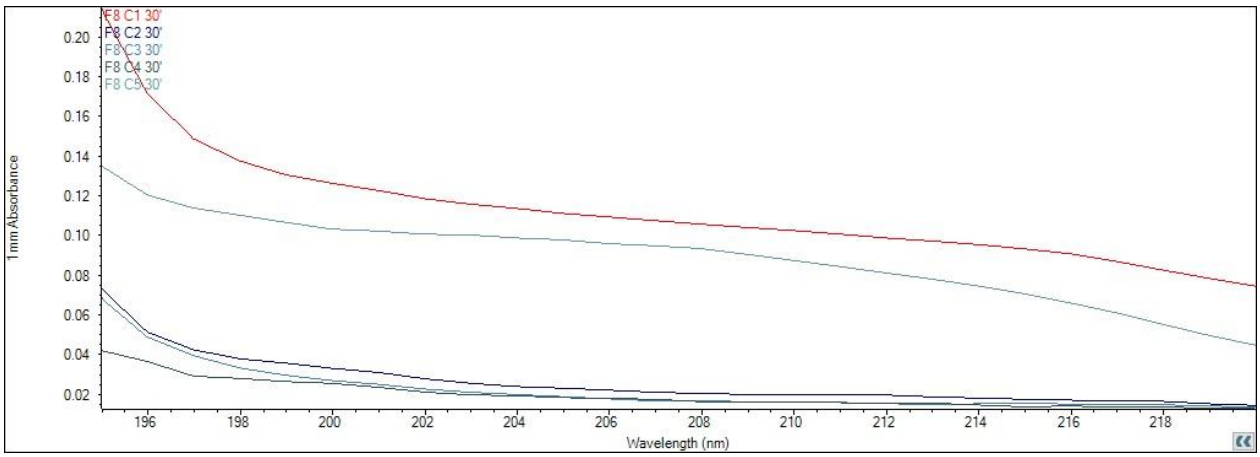
Spectra



Strat-M® Membrane – GlcNAc + Glycerol + Propylene Glycol + Water (1:1:1)

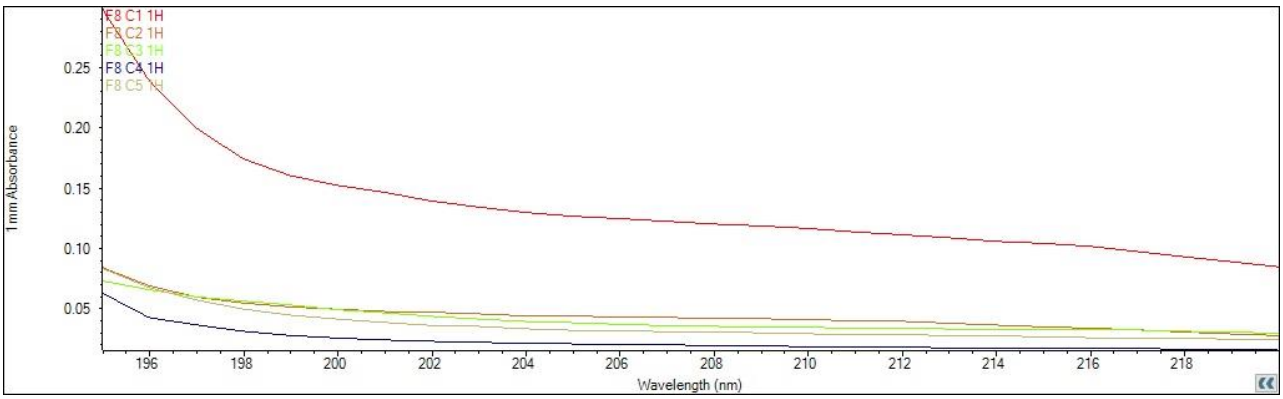
After 0.5h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10808	0.10806	0.10845
$\mathbf{m}_{a,max}$	0.002162	0.002162	0.002169
$\mathbf{A}_{bl}$ (220 nm)	0.074	0.014	0.013
$\mathbf{A}_{max,a,t}$ (202 nm)	0.118	0.027	0.022
$\mathbf{c}_{a,t}$	45.22	25.58	23.04
$\mathbf{m}_{a,t}$	316.54	179.06	161.28
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	14.547	8.387	7.647

Spectra



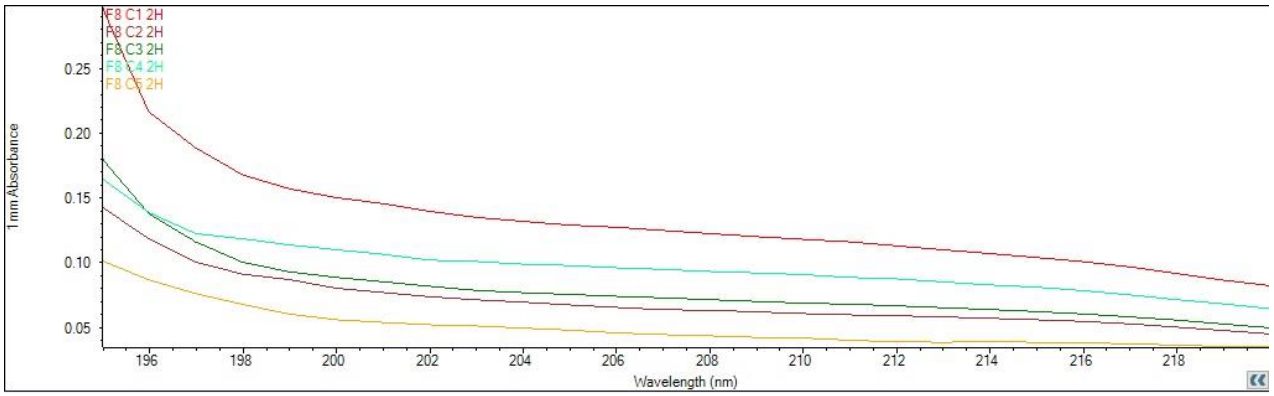
After 1h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10808	0.10806	0.10845
$\mathbf{m}_{a,max}$	0.002162	0.002162	0.002169
$\mathbf{A}_{bl}$ (220 nm)	0.084	0.027	0.029
$\mathbf{A}_{max,a,t}$ (202 nm)	0.139	0.046	0.043
$\mathbf{c}_{a,t}$	52.19	29.38	26.21
$\mathbf{m}_{a,t}$	365.33	205.66	183.47
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	16.789	9.633	8.699

Spectra



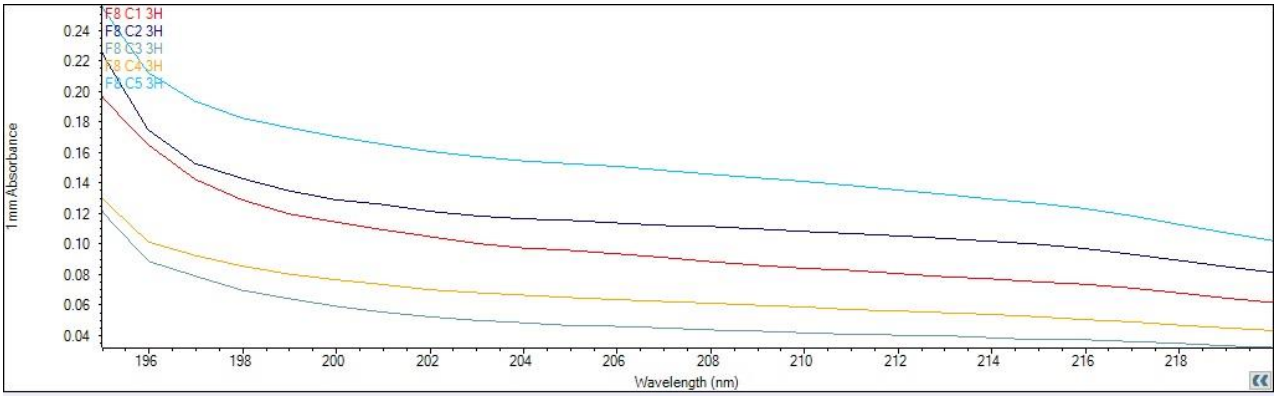
After 2h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10808	0.10806	0.10845
$m_{a,max}$	0.002162	0.002162	0.002169
A_{bl} (220 nm)	0.082	0.044	0.049
$A_{max,a,t}$ (202 nm)	0.139	0.073	0.081
$c_{a,t}$	53.46	35.72	37.62
$m_{a,t}$	374.22	250.04	263.34
$m_{a,t}/m_{a,max}$	17.198	11.711	12.486

Spectra



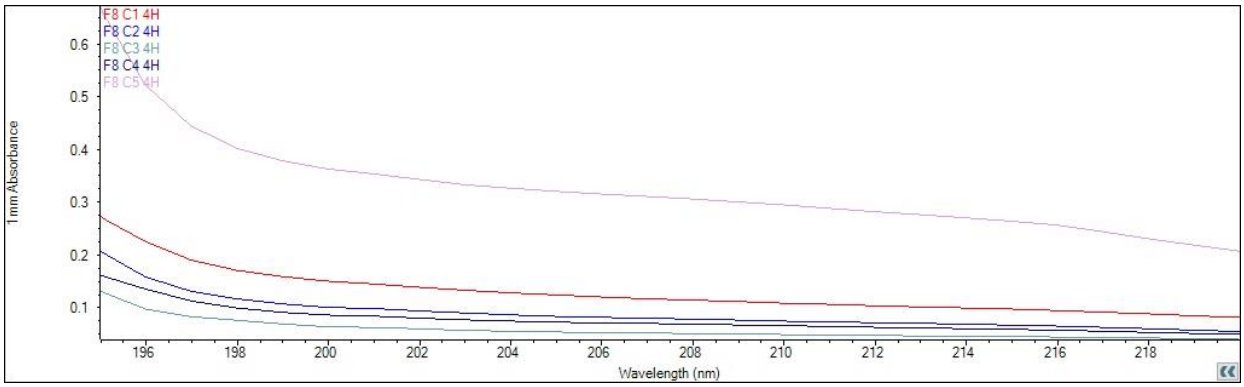
After 3h			
	Cell 1	**Cell 2**	**Cell 3**
m_{form}	0.10808	0.10806	0.10845
$m_{a,max}$	0.002162	0.002162	0.002169
A_{bl} (220 nm)	0.061	0.081	0.031
$A_{max,a,t}$ (202 nm)	0.104	0.121	0.052
$c_{a,t}$	44.59	42.69	30.65
$m_{a,t}$	312.13	298.83	214.55
$m_{a,t}/m_{a,max}$	14.344	13.997	10.173

Spectra



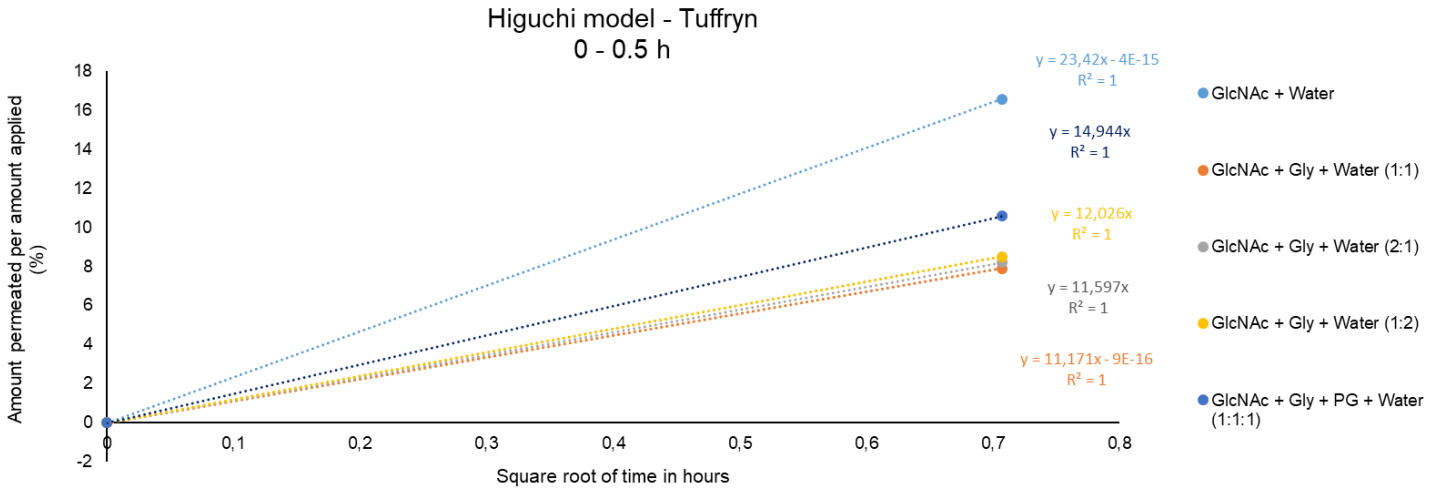
After 4h			
	Cell 1	**Cell 2**	**Cell 3**
$\mathbf{m}_{form}$	0.10808	0.10806	0.10845
$\mathbf{m}_{a,max}$	0.002162	0.002162	0.002169
$\mathbf{A}_{bl}$ (220 nm)	0.08	0.053	0.038
$\mathbf{A}_{max,a,t}$ (202 nm)	0.137	0.092	0.058
$\mathbf{c}_{a,t}$	53.46	42.05	30.01
$\mathbf{m}_{a,t}$	372.22	294.35	210.07
$\mathbf{m}_{a,t}/\mathbf{m}_{a,max}$	17.106	13.787	9.961

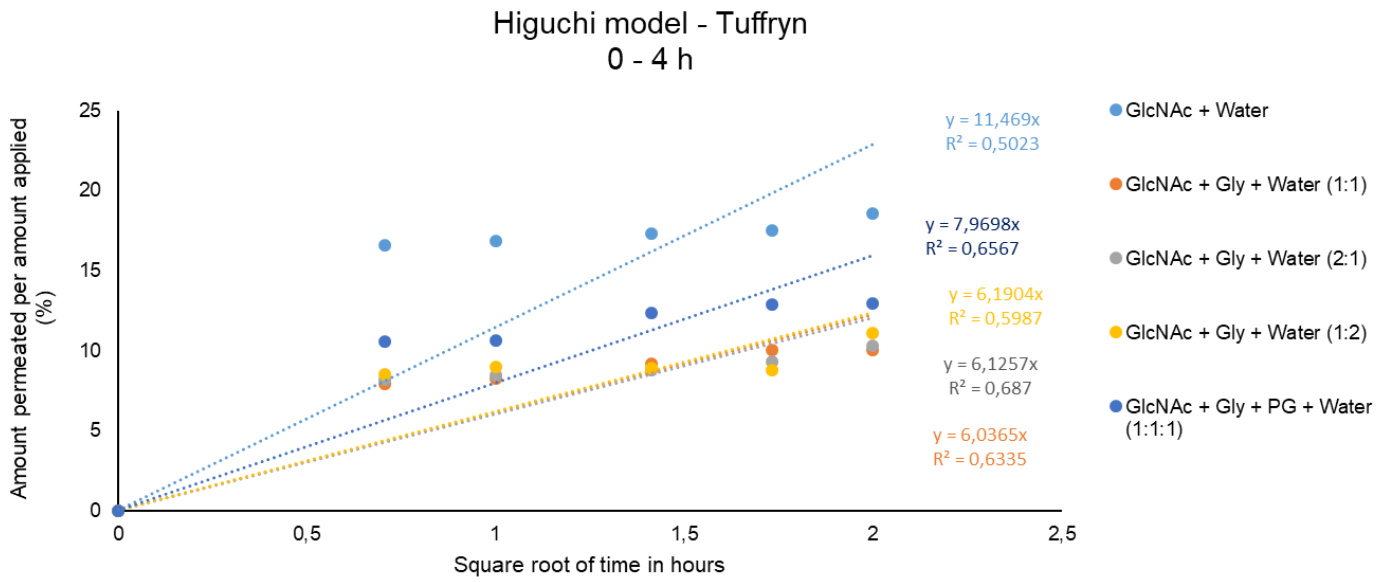
Spectra

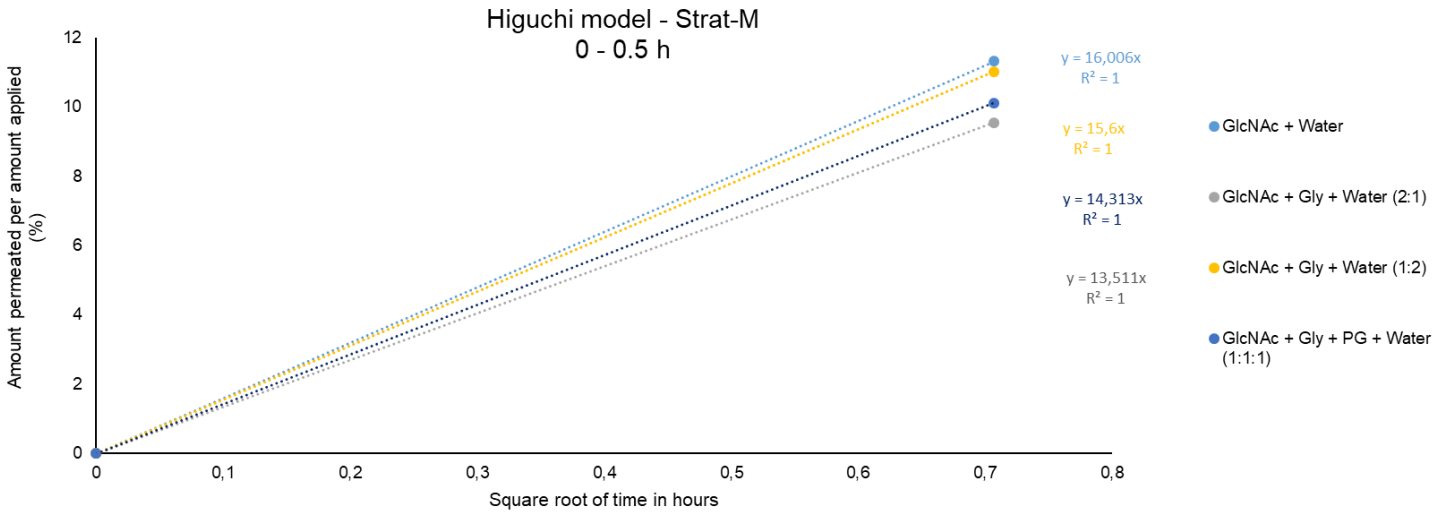


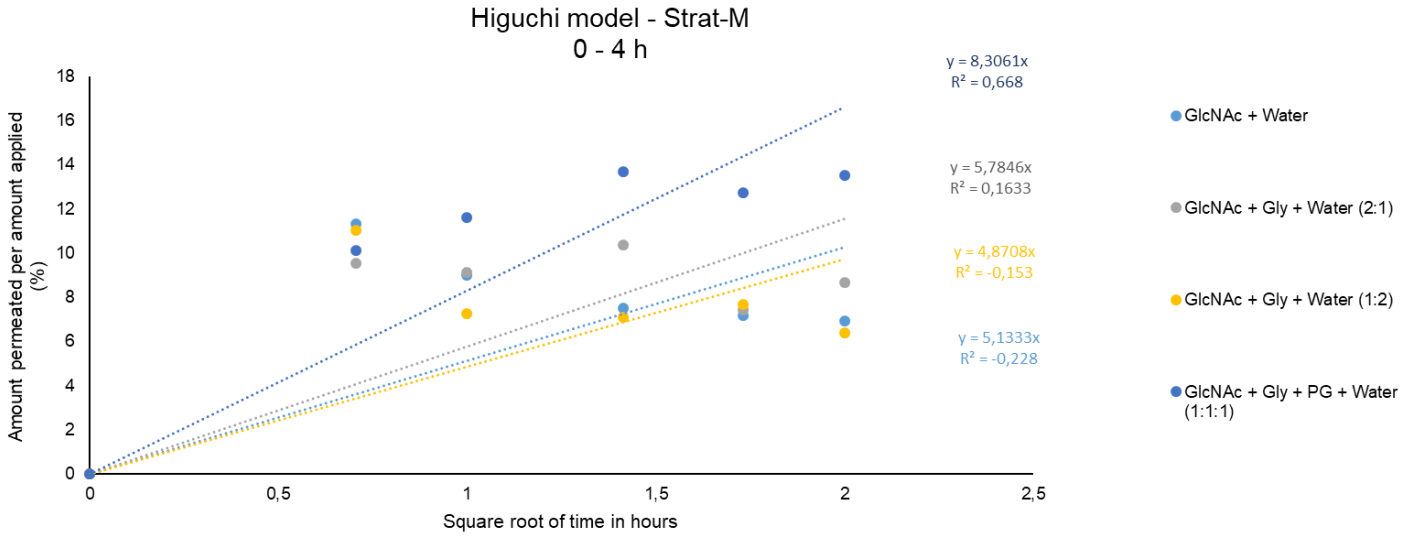
m_{form}	Mass of the formulation on the top of the disk/membrane
$m_{a,max}$	Mass of the active in the formulation on the top of the disk/membrane at t = 0 s
A_{bl}	Absorbance of the base line (220 nm)
$A_{max,a,t}$	Absorbance of the active after t release time (202 nm)
$c_{a,t}$	Concentration of the active after t release time
$m_{a,t}$	Mass of the active after t release time
$m_{a,t}/m_{a,max}$	Ratio of the mass of the active after t release time and the mass of the active in the formulation on the top of the disk/membrane at t = 0 s

13. Appendix II






Higuchi model - Strat-M
0 - 0.5 h
Amount permeated per amount applied (%)
Square root of time in hours
y = 16,006x
R² = 1
y = 15,6x
R² = 1
y = 14,313x
R² = 1
y = 13,511x
R² = 1
GlcNAc + Water
GlcNAc + Gly + Water (2:1)
GlcNAc + Gly + Water (1:2)
GlcNAc + Gly + PG + Water (1:1:1)


Higuchi model - Strat-M
0 - 4 h
Amount permeated per amount applied (%)
Square root of time in hours
y = 8,3061x
R² = 0,668
y = 5,7846x
R² = 0,1633
y = 4,8708x
R² = -0,153
y = 5,1333x
R² = -0,228
GlcNAc + Water
GlcNAc + Gly + Water (2:1)
GlcNAc + Gly + Water (1:2)
GlcNAc + Gly + PG + Water (1:1:1)