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Studies of the corneocytary pathway across the Stratum corneum. Part I: Diffusion of amino acids into the isolated corneocytes

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Amino acids (AAs), important constituents of natural moisturizing factors (NMFs) of the skin are decreased in diseased conditions such as psoriasis and atopic dermatitis. No study so far investigated the uptake of AAs into isolated corneocytes (COR). The present study was performed using 19 AAs, including taurine (TAU), to measure their amount diffused into the COR and binding of these AAs to keratin. Incubation of alanine, aspartic acid, asparagine, glutamine, glutamic acid, histidine, proline, serine and TAU with the isolated COR showed uptake after 24 h of 51.6, 95.4, 98.6, 94.1, 95.6, 90.1, 94.6, 72.9 and 57.8 %, respectively, into the COR but no binding with keratin. Uptake of TAU was validated by time dependent *in-vitro* diffusion models \`without COR and \`with COR´. The time dependent curve fitting showed that in *in-vitro* diffusion model \`without COR´ there was no change in the total concentration of TAU until 72 hours, while in diffusion model \`with COR´ the total conc. decreased to 37.8 % after 72 hours. The Pearson´s correlation coefficient \`r´ between the conc. curves of both *in-vitro* diffusion models was -0.54 that was an evidence of significant amount of TAU uptake by the COR. AAs as part of the NMFs have a great potential to be diffused into the COR. This property of the AAs can be employed in further dermatological research on diseased or aged skin conditions with NMFs deficiency.

1. Introduction

Stratum corneum (SC) is the outer most layer of the epidermis comprising 18-20 layers of enucleated cells called corneocytes (COR) which are formed by the process of desquamation (Piérard et al. 2000; McGrath et al. 2008; Jepps et al. 2013) from keratinocytes. COR are surrounded by a protein shell called cornified envelop (Kalinin et al. 2002; Mojumdar et al. 2017) along with a covalently bound lipid monolayer (Elias et al. 2014). The layers of COR are (Sylvestre et al. 2010) embedded in multilamellar lipid matrix or intercellular lipid composed of ceramides (40 mol %), free fatty acids (30 mol %), cholesterol (30 mol %) and its derivatives (Elias et al. 2014). COR are filled with keratin and histidine-rich protein, called filaggrin, which is bound to the keratin. Filaggrin produces natural moisturizing factors (NMFs) confined inside COR (McGrath et al. 2008). Various studies have reported the presence of NMFs in the epidermis as hydrophilic compounds consisting of amino acids (AAs), its derivatives such as pyrrolidone carboxylic acid and other compounds such as lactates, sugar, peptides, urea and also inorganic salts of sodium, potassium, calcium and magnesium (Rawlings et al. 1994; Harding et al. 2000). Already reported AAs include alanine, asparagine, arginine, aspartic acid, cysteine, citrulline, glutamine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, valine and taurine (TAU) (Jacobson et al. 1990; Caspers et al. 2001; Sylvestre et al. 2010; Visscher et al. 2011; Joo et al. 2012; Jassoy et al. 2015). These compounds of the NMFs, particularly the AAs, play an important role as humectants, and attract the water into COR even at a relative humidity of as low as 50 %. Hence, NMFs maintains the hydration status of the SC (White-Chu and Reddy 2011) reduction in the level of NMFs leads to dry skin in conditions such as atopic dermatitis, psoriasis and ichthyosis vulgaris (Takahashi and Tezuka 2004; Kwan et al. 2012; Levin et al. 2013; Takahashi et al. 2014).

Both lipids and COR of the SC are organized to maintain the barrier function of the skin. Many of the researchers described this arrangement of COR and lipids as ‘brick and mortar’ (respectively) model (Hachem 2006; White-Chu and Reddy 2011; Anissimov et al. 2013) which represents the heterogeneous nature of the SC (Williams and Barry 2004; Chen et al. 2009; Bolzinger et al. 2012) consisting of the polar COR and non-polar SC highly ordered lipid matrix.

Drugs applied dermally have mainly to cross the SC in order to reach the living epidermis and the dermis. It is well accepted in the literature that drug penetration across the SC occurs intercellularly *via* the lipid lamella which will naturally favour only the penetration of lipophilic molecules. In contrast, the transcellular or corneocytary pathway *via* COR and the corneodesmosomes is not well studied to date. However, this pathway would allow the diffusion of hydrophilic drugs to pass through the SC (Barry 1987; Moser et al. 2001). The transcellular route has limitations and it is not clear how to influence this pathway because COR, accompanying this route, act as reservoir for most of the hydrophilic molecules. The keratin, present inside COR, can play a vital role in the reservoir property of these cells due to their binding affinity to the hydrophilic molecules. Thus, the molecules passing COR are entrapped inside COR and the time taken by the molecules to get into and out of COR ultimately effects

Abbreviations: NMFs: natural moisturizing factors, AAs: Amino acids, PBS: Phosphate buffer saline; SC: Stratum corneum, LC-ESI-MS/MS: Liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry, ACC: Acceptor compartment; DON: Donor compartment, RC: Regenerated cellulose, TAU: Taurine, COR: Corneocytes, DDW: Double distilled water, Soln: Solution.

the total transfer rate, of dermal drugs (Sznitowska et al. 1998; Heard et al. 2003; Seif and Hansen 2012; Patel et al. 2015; Choe et al. 2017).

To date. various studies have been conducted for the development of *in-vitro* diffusion models for hydrophilic molecules across the SC following transcellular route (Wang et al. 2006; Marquez-Lago et al. 2010; Xiao and Imhof 2012; Couto et al. 2014). However, none of the studies used the isolated COR for predicting the diffusion of AAs into COR.

Therefore, the objective of this work was to determine the uptake of AAs by the isolated COR, and binding of AAs with keratin powder. Additionally *in-vitro* diffusion models `without COR´ and `with COR´ were developed to validate the uptake of TAU (model AA), by COR. TAU (2-aminoethanesulfonic acid) was selected as model AA because of its hygroscopic nature compared to other AAs of the NMFs and additionally, it has osmoregulatory and antioxidant properties. In one of the studies it was also reported that TAU stimulates the synthesis of all three barrier lipids (ceramides, fatty acid and cholesterol) in reconstructing epidermis (Boelens et al. 2003; Odetti et al. 2003; Nishimura et al. 2009).

Table 1. Stability studies of AAs including TAU (n = 3, ± SD).

AA	Initial concentration (μM)	Concentration(μM) after 24 hours ±SD	AA	Initial concentration (μM)	Concentration(μM) after 24 hours ±SD
Aln	120 ± 1.8	118 ± 1.5	Pro	70 ± 0.05	69 ± 0.5
Arg	70 ± 1.9	72 ± 0.5	Ser	310 ± 0.0	311 ± 0.9
Asp	100 ± 0.5	101 ± 1.2	TAU	108 ± 0.1	24 h- 99 ± 0.7
Asn	120 ± 0.9	105 ± 1.6			48 h- 101± 1.1
Gln	110 ± 1.4	108 ± 0.9			72 h- 97± 1.7
Glu	100 ± 1.5	99.9 ± 0.4	Thr	90 ± 0.8	88 ± 1.2
Gly	90 ± 0.8	92 ± 0.7	Trp	70 ± 0.3	71 ± 0.5
His	64 ± 1.9	65 ± 0.8	Tyr	210 ± 0.1	208 ± 0.7
Ileu	110 ± 1.1	110 ± 0.5	Val	80 ± 0.9	80 ± 0.9
Leu	150 ± 0.8	149 ± 1.3	Cit	180 ± 0.8	177 ± 0.5
Phe	150 ± 0.8	148 ± 1.6			

2. Investigations, results and discussion

2.1. Stability studies of AAs and TAU

Incubation of selected AAs after 24 h under the conditions mentioned resulted in no degradation. TAU after 72 h was also observed to be stable as shown in Table 1. As the results show (Table 1) all the AAs soln. in DDW are stable for 24 h at 32 °C and 11.8 % humidity. TAU incubated for 72 h was also observed to be stable with only a negligible reduction in the amount that was present at the initial time. So, none of the experimental conditions resulted in the reduction of the concentration during the interaction of AAs and TAU with the isolates COR and keratin experiment. Hence, all the studied AAs were stable at the selected conditions.

2.2. Uptake of AAs and TAU by the isolated COR

The results presented in Table 2 are showing the initial concentration of each AAs and TAU along with the concentration left after 24 h. Table 2 also shows the percentage of each AAs and TAU uptake by the isolated COR. Alanine, aspartic acid, asparagine, glutamine, glutamic acid, histidine, proline, serine and TAU showed uptake rates of 51.6, 95.4, 98.6, 94.1, 95.6, 90.1, 94.6, 72.9 and 57.8%, respectively. AAs are a chemically diverse group of molecules and thus exhibit different behaviour under the same experimental conditions. So, nine out of 19 AAs showed uptake into the isolated COR, while the remaining AAs either had none or very negligible amount diffused into COR. All of these AAs with higher uptake into the isolated COR belong to the non-essential class of AAs except histidine and aspartic acid. Non-essential AAs are those AAs which are synthesized in the human body. The reason why they were showing a higher uptake may be that they are more natural to human body with respect to pH, osmotic properties of the cellular content. Asparagine which showed the maximum uptake (98.6 %) into the isolated COR, is a polar AA. The other AAs which showed uptake are also polar except alanine and proline. COR serve as absorption depot for hydrophilic molecules which was demonstrated by visualization of COR using TEM and photon microscopy (Boddé et al. 1991; Yu et al. 2003; Jacobi et al. 2006) so is the case with AAs including TAU. A lot of efforts have been done in research related to prediction of permeability of hydrophilic drugs across SC. However, to the best of our knowledge, none of the studies was carried out using AAs and TAU uptake into the isolated COR. However, reported measurement data of more than 50 hydrophilic molecules, other than AAs and TAU, of dermatological value for predicting their skin permeability is available (Potts and Guy 1992; Wilschut et al. 1995; Mitragotri 2003; Lian et al. 2008; Chen et al. 2009).

Table 2. Uptake of AAs and TAU into the isolated COR after 24 hours of incubation (n=3, ± SD).

AA	Initial Concentration (μM)	Concentration (μM) left after 24 hours ± SD	% age of AA uptake by the corneocytes
Aln	120 ± 1.8	56 ± 9.4	51.6
Arg	70 ± 1.9	55 ± 2.4	16.9
Asp	100 ± 0.5	4 ± 2.3	95.4
Asn	120 ± 0.9	2 ± 0.6	98.6
Gln	110 ± 1.4	6 ± 1.6	94.1
Glu	100 ± 1.5	4 ± 1.6	95.6
Gly	90 ± 0.8	66 ± 11.1	27.1
His	64.0 ± 1.9	6.1 ± 38	90.1
Ileu	110 ± 1.1	102 ± 5.5	7.5
Leu	150 ± 0.8	137 ± 3.2	8.9
Phe	150 ± 0.8	119 ± 8.3	20.7
Pro	70 ± 0.05	4 ± 24	94.6
Ser	310 ± 0.0	85 ± 8.7	72.9
TAU	108 ± 0.1	45.4 ± 20.8	57.8
Thr	90 ± 0.8	84.3± 23.7	6
Trp	70 ± 0.3	69 ±9.6	1.2
Tyr	210 ± 0.1	152 ± 8.5	27
Val	80 ± 0.9	72 ± 10.2	9.8
Cit	180 ± 0.8	170 ± 2.9	5.2

Table 3. Interaction of selected AAs and TAU with keratin after 24 hours of incubation (n = 3, ± SD).

AA	Initial Concentration (μM)	Concentration(μM) after 24 hours ± SD	AA	Initial concentration (μM)	Concentration (μM) after 24 hours ± SD
Interaction with Keratin					
Ala	128.0 ± 1.5	121 ± 5.5	His	134.8 ± 0.9	140 ± 5.5
Asp	120.4 ± 1.9	132 ± 10.3	Pro	93.3 ± 0.5	89.5 ± 6.3
Asn	101.1 ± 0.6	121 ± 11.2	Ser	70.4 ± 0.7	70.9 ± 0.5
Gln	97.7 ± 0.8	103 ± 1.6	TAU	99.8 ± 1.0	95.1 ± 10.0
Glu	94.3 ± 1.2	94.6 ± 0.0			

2.3. *Binding of AAs to keratin*

Incubation of selected AAs including TAU with keratin powder resulted in no change in concentraition after 24 h with DDW as medium (see Table 3). It has been mentioned that when hydrophilic molecules are absorbed into COR, they bind to keratin (Heard et al. 2003; Seif and Hansen 2012; Patel et al. 2015). In contrary, our results showed that none of the AAs and TAU showed binding with keratin.

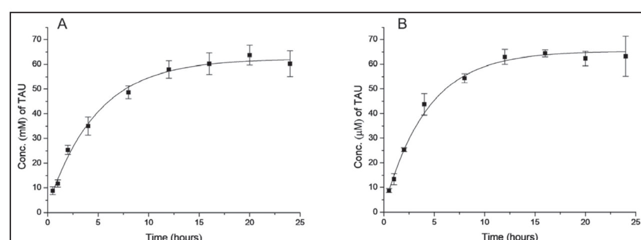

Fig. 1: Conc. profile of TAU in ACC compartment in the in-vitro diffusion model \`without COR´ (A) (n = 3, RSD = ≤ 10 %). and in the in-vitro diffusion model \`with COR´ (B) after 24 hours (n = 3, RSD = ≤ 35 %).

Table 4: Percentage comparison of total conc. of TAU left in both compartments of *in-vitro* diffusion models \`without COR´ and \`with COR´ at different time points, (n = 3)

Time points (hours)	\`Without COR´	\`With COR´
	% of the conc.	
C_0	100	100
C_{24}	99	94
C_{48}	94	47
C_{72}	94	37.8

2.4. *In-vitro diffusion models of TAU \`without COR´ and \`with COR´*

As *in-vitro* diffusion model \`without COR´ the DON was filled with standard solution of TAU while ACC contained only DDW, as a diffusion medium, separated by a porous membrane. Due to the continuous stirring supplied to diffusion cells TAU was diffused from DON to ACC along with a concentration gradient between both compartments. TAU concentration increased exponentially in the ACC compartment for the first 12 h (see Fig. 1A), and then reached at dynamic equilibrium. The equilibrium was a point where the concentration of TAU distributed between ACC and DON was exactly half of the total initial concentration \`C_0. The experiment was continued for the next 72 h, and no change in the dynamic equilibrium was observed so was the concentration in the respective subcompartments. The reduction in the total TAU concentration was not significant (C_{24} = 100 %, C_{48} = 94 %, C_{72} = 94 %, Table 4).

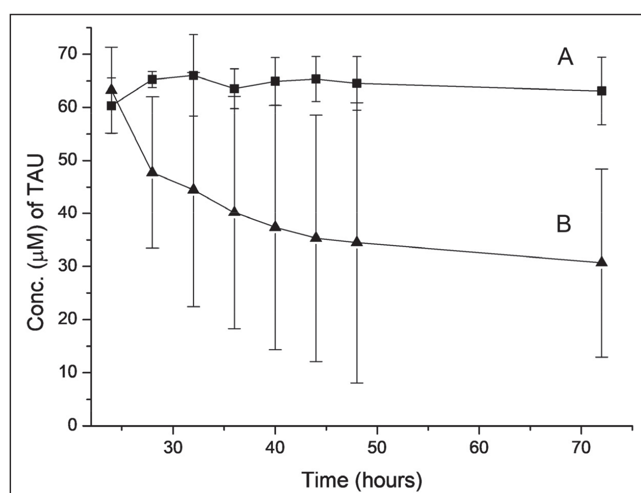

Fig. 2: Conc. profile of TAU in ACC compartment in the *in-vitro* diffusion model \`without COR´ (A) (n = 3, RSD < 15 %) and in the *in-vitro* diffusion model \`with COR´ (B) for 72 hours (n = 3, RSD < 70 %).

While in the *in-vitro* diffusion model \`with COR´ we expected the change in diffusion of TAU across membrane owing to the presence of the isolated COR in the DON in addition to TAU standard solution under the same experimental conditions. However, the same exponential increase of TAU concentration in the ACC compartment was observed as in the model \`without COR´. While the equilibrium was reached after 8 h (Fig. 1B) this was a shorter time when compared with the equilibrium time of *in-vitro* diffusion \`without COR´. The literature reported that COR swell when immersed or come in contact with water for a longer period of time (Richter et al. 2001). The TAU concentration in DON was increased due to the reduction of water, which was taken up by the isolated COR during their swelling process. So, this shorter duration to reach equilibrium was attributed to the more concentrated TAU solution in the DON, which resulted in faster movement from DON to ACC. For the next 24 h, there was no change in the dynamic equilibrium but after 24 h a sudden decrease in TAU concentration occurred both in the ACC as well as in the DON (Fig. 2 curve B).

After 24 h the isolated COR reached a maximum swollen condition, which resulted in changed morphology of COR and hence, increased permeability for TAU inside COR. After 24 h, the total concentration of TAU in ACC and DON was C_{24} = 94 % of the initial concentration. and at 48 and 72 h it was measured to be as C_{48} = 47 % and C_{72} = 37.8 %, respectively (see Table 4). This means that after 24 h, TAU started to diffuse into the isolated COR, and continued until 72 h. In 72 h, the maximum concentration of TAU was diffused into the isolated COR which exhibited its maximum uptake capacity. The equilibrium was again established with the remaining (C_{72} = 37.8 %) TAU in both subcompartments.

When comparing the time *vs* conc. curves A and B (Fig. 2) of *in-vitro*\`without COR´ and \`with COR´, it is clear that there is a significant change in concentration observed after 24 h in the *in-vitro* diffusion model \`with COR´ as shown in Fig. 2. This difference was quantified from both curves by calculating the Pearson´s correlation coefficient (r) using corrected concentrations between 24 to 72 h for *in-vitro* diffusion \`without COR´and \`with COR´ The value of \`r´ calcd. was -0.54. The negative value is an indication of an opposite concentration trend between both curves indicating that either of the curves is moving in the opposite direction. The higher the negative \`r´ value between 0 and -1 is, the greater is the uptake of the test molecule by COR.

The results show that COR exhibit high resistance for the transport of AAs across the transcellular route due to their reservoir property. This reservoir property can be utilized positively for targeted delivery of AAs in dermatological disorders with the NMFs deficiency to replenish the effected COR. The measurement of \`r´ value based on these *in-vitro* diffusion models is a useful prediction tool and can be used in evaluating molecules with significant uptake into COR.

Furthermore, these models can be used to evaluate various controlled release matrix systems used in nanocarrier formulations as well as for the identification of penetration enhancers showing maximum diffusion into COR.

3. Experimental

3.1. *Materials*

Standard AAs (l-alanine, l-asparagine, l-arginine, l-aspartic acid, l-citrulline, l-glutamine, l-glutamic acid, l-glycine, l-histidine, l-isoleucine, l-leucine, l-phenylalanine, l-proline, l-serine, l-threonine, l-tyrosine, l-tryptophan, TAU, l-valine) chloroform and methanol were purchased from VWR (International GmbH, Darmstadt, Germany). NaCl, KCl, LiCl (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) anhydrous Na_2HPO_4 and KH_2PO_4 (Merck KGaA, Darmstadt, Germany) were used for the preparation of phosphate buffer saline (PBS) (pH 7.4). All of the above chemicals used were of analytical grade. Keratin powder, prepared from the ram´s horn, was obtained from Chroma Chemical Corp, Suqian, China. Dry spectra /pore 1, regenerated cellulose (RC) synthetic dialysis membrane, 6- 8 kDa, ø 1.8 - 2.4 nm product of spectrum lab

Table 5: Composition of *in-vitro* diffusion models `without COR´ and `with COR´

Model	Without COR	With COR
	Composition	
DON	TAU (100µM)	TAU+COR (100µM+ 10mg/ml)
ACC	DDW	DDW

was obtained from VWR, International GmbH, Darmstadt, Germany. The membrane was cut into circular form with total area of 15.9 cm^2. Norvaline (analytical grade) which was used as internal standard (100 µM), Fmoc/Cl and, standard AA were obtained from Sigma-Aldrich (St. Louis, MO, USA), syringes and needles (1 ml) from B. Brown (Melsungen, Germany). Double distilled water (DDW) was obtained from water distillation plant of the Institute of Pharmacy of the MLU.

3.2. Preparation of AAs free isolated COR

The left over skin after pedicure treatment was obtained from a foot-care clinic (Neustadt Zentrum, Halle, Germany). The obtained material (1 g of skin) was crushed with the help of pestle and mortar with twice addition of liquid nitrogen. After crushing, the material was washed five times with PBS. After removal of dirt, the material was subjected to extraction of intercellular lipids by adding 150 ml of chloroform/methanol (2/1, v/v) mixture and stirred with the magnetic stirrer at 1200 rpm for 48 h. The mixture was then filtered through a sintered glass frit R4 under vacuum. The de-lipidized COR were collected and again combined with 150 ml of fresh chloroform/methanol (2/1, v/v) mixture and subjected to stirring using an IKA Ultra-Turrax (NeoLab Migge GmbH, Heidelberg, Germany) homogenizer with S18N- 19G dispersing element, at 11000 rpm for 30 min and filtered through a sintered glass frit under vacuum. The second mixture step helped in detachment of COR from each other and resulted in the isolated COR that were sieved (mesh no. = 40, pore size 420 µm).
COR (1 mg/10 ml) in DDW were incubated at 46 °C for 24 h to extract naturally present AAs in the isolated COR. After 24 h, the COR were centrifuged at 5,000 x g for 10 min. The supernatant, which contained extracted AAs was discarded, suspended COR were collected and used for further study.

3.3. Preparation and stability studies of standard solutions of AAs

Standard solution (100 µM) of each of the AAs and TAU were prepared in DDW (pH, 6.8) and concentration was determined at different time points for 24 h for all AAs, for 24, 48 and 72 hours for TAU to observe the stability of the respective solution at 32 °C and 11.8 % humidity. Humidity was established by the use of the saturated salt solution of LiCl.

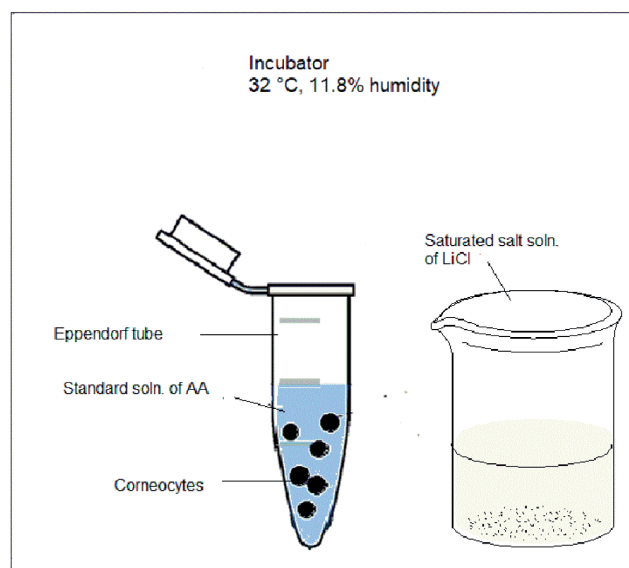

Fig. 3: Schematic diagram for experimental design for the uptake of AAs/TAU into the isolated COR.

3.4. Uptake of AAs and TAU into the isolated COR

Standard solutions of all individual AAs including TAU (100 µM) were incubated separately with the isolated COR (10 mg/ml) at 32 °C for 24 h at 11.8 % humidity, each in a separate Eppendorf tube (Eppendorf AG, Hamburg, Germany) (see Fig. 3). After 24 h, samples were centrifuged (5000 x g, 10 min), the supernatant was analysed for quantity of AAs. The experiment was repeated three times.

3.5. Binding of AAs with keratin

Standard solutions of selected AAs (Ala, Asp, Asn, Gln, Glu, His, Pro, Ser) and TAU (100 µM) were incubated with keratin (10 mg/ml) at 32 °C for 24 h at 11.8 % humidity, each in a separate Eppendorf tube. After 24 h, the sample was filtered using syringe filters (0.45 µm), to remove keratin, and analysed for remaining concentrations of AAs and TAU. The experiment was repeated three times.

3.6. In-vitro diffusion models of TAU `without COR´ and `with COR´

In-vitro diffusion models `without COR´ and `with COR´, which are a modification of diffusion model previously described by Neubert and Fürst (1989) (Fig. 4), were used to study the diffusion profile of TAU uptake into the isolated COR. Both *in-vitro* diffusion models `without COR´ (TAU 100 µM) and `with COR´ (TAU 100 µM+10 mg/ml COR) (Table 5) comprised three diffusion cells each consisting of acceptor (ACC) and donator (DON) compartments containing 20 ml of diffusion medium (DDW) of pH 6.8. These compartments were immersed approximately 90 % in a water bath and were subjected to continuous stirring by integrated vibration system. The temperature of the water bath was maintained at 32 °C with inbuilt temperature regulator. DON and ACC were

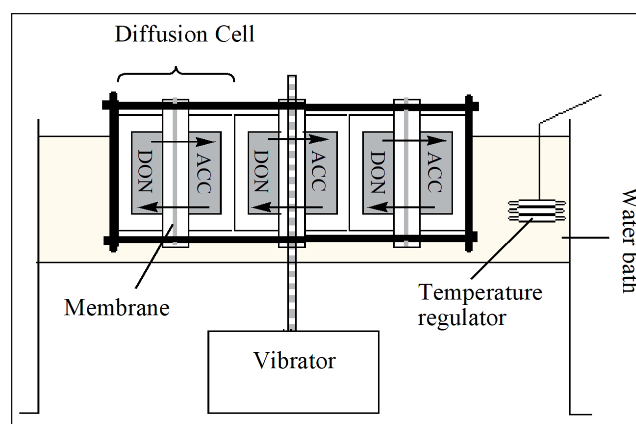

Fig. 4: Diffusion apparatus used in *in-vitro* diffusion models `without COR´ and `with COR´.

separated by a hydrophilic dialysis synthetic membrane (RC) with total area of 15.9 cm^2 allowing the transport of TAU from DON to ACC compartment. Prior to the experiment the membrane was soaked in DDW for 30 min. Sample (1 ml) was drawn at specific time points (0.5, 1, 2, 4, 8, 12,…., 48 and 72 h) from ACC and (24, 48 and 72 h) from DON of both `without COR´and `with COR´models. After withdrawal samples were stored at 4 °C for one week and at -20 °C for longer duration. The withdrawn sample volume was refilled at every time to keep the total volume of the compartments constant. TAU diffused from DON through the dialysis membrane into the ACC following simple passive diffusion along the conc. gradient governed by FICK`s first law of diffusion as follows:

$$a_{DON} = -DA\frac{dc}{dx} = -DA\frac{(c_{DON} - c_{ACC})}{\Delta x} \quad (1)$$

The total conc. of TAU will be equal to the sum of conc. distributed equally between both compartments at time when equilibrium is achieved. `$C_{eq}$´ is conc. at equilibrium that is half of the initial conc. `C_0´ if the volumes of DON and ACC are equal.

$$c_{eq} = \frac{C_0}{2} \quad (2)$$

with same volume and pH in both DON and ACC, the separating membrane is the only factor offering a rate-limiting barrier for the transport of TAU molecules across DON and ACC (Fürst et al. 1975, 1980; Neubert 1978; Neubert and Fürst 1989; Enderle 2012).
We have used `C_{cor}´ (corrected conc.) for curve fitting which was calcd. using following formula:

$$C_{cor} = C_{tn} \pm C_{tn-1}\cdot\frac{V_s}{V_{ACC}} \pm C_{tn-2}\cdot\frac{V_s}{V_{ACC}} \ldots \pm C_{t1}\cdot\frac{V_s}{V_{ACC}} \quad (3)$$

where, `$C_{tn}$´ is conc. at final time point, `C_{tn-1}´ is conc. at point former to the last time point, `$V_s$´ is sample volume. Pearson´s correlation coefficient was calcd. using corrected conc. of *in-vitro* diffusion models `without COR´and `with COR´between the time C_{24} to C_{72}, to observe the relative difference in the conc. curves of *in-vitro* diffusion models.

3.7. Analytics

AAs and TAU were quantified by LC-ESI-MS/MS as its FMOC-derivative after adding 10 mM norvaline as internal standard, according to the protocol for AAs determination published by Ziegler and Abel (2014). MS parameters describing the MRMs for AAs/TAU are available from the authors on request.

3.8. Data analysis

Curve fitting was done by OriginPro 8.5.1. SR2 b315 (OriginLab cooperation, Northampton, MA, USA). Statistical analysis and Pearson´s correlation coefficient was determined using Microsoft® Excel, 2013 (Santa Rosa, California, USA).

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